The Role of Estrogen Signaling in the Induction, Specification, and Proliferation of Hematopoietic Stem Cells

The Role of Estrogen Signaling in the Induction, Specification, and Proliferation of Hematopoietic Stem Cells

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Abstract

Hematopoietic Stem Cells (HSCs) are characterized by their ability to both self-renew and give rise to all lineages of the blood system. A recent chemical genetic screen identified 17β-estradiol (estrogen) as a novel modifier of the expression of the conserved HSC markers *runx1* and *cmyb* in the Aorta-Gonad-Mesonephros of developing zebrafish. Exposure to exogenous estrogen during the development of the hematopoietic niche impeded specification of hemogenic endothelium and the subsequent emergence of HSCs via antagonism of somitic-derived VEGF signaling. Conversely, inhibition of endogenous estrogen activity increased the number of functional HSCs present in the embryo and resulted in higher expression of VEGF target genes, suggesting that endogenous estrogen acts to define the ventral limit of VEGF activity and hemogenic endothelial specification.

In contrast, when embryos were exposed to estrogen after niche specification, markers of HSCs were increased, indicating that estrogen has a biphasic effect on HSC formation; this effect appears to be at least partially mediated by enhanced cell cycling of the HSC population. Estrogen exposure during primitive erythropoiesis likewise increased the number of erythroid progenitors in the embryo, but their maturation into functional erythrocytes was impaired. Inhibition of erythrocyte maturation is also conserved in a mammalian model of *in utero* excess estrogen, causing propensity for embryonic lethality.

Treatment of adult zebrafish with exogenous estrogen after ablation of the hematopoietic system by irradiation revealed that elevated estrogen levels improved hematopoietic regeneration. Consistent with a role for hormonal regulation of HSC homeostasis, accelerated recovery of hematopoietic stem and progenitor numbers was observed in female fish compared to males, suggesting an endogenous difference in regenerative capacity between the sexes. Together, these data identify multiple distinct roles for estrogen in HSC biology and indicate it is a physiologically relevant regulator of HSC development and homeostasis.

Table of Contents

Citations to Published/Submitted Work

Parts of Chapter 1 are in press:

> **Carroll KJ** and North TE. Oceans of Opportunity: Exploring Vertebrate Hematopoiesis in the Zebrafish. (2014) *Experimental Hematology. In Press.*

Parts of Chapter 2 are in press:

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Appendix I is in revision:

> Esain V, **Carroll KJ**, Cortes M, Liu S, Kwan W, Frechette GM, Sheward L, Goessling W, North TE. Cannabinoid Receptor-2 Signaling Regulates Embryonic Hematopoietic Stem Cell Function via ProstaglandinE2 and P-selectin in Zebrafish. (2014) *Blood. In Revision*.

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Chapter 1:

Introduction

Identification and Characterization of the Hematopoietic Stem Cell

Hematopoiesis is the process by which all the blood cells of an organism are formed. The hematopoietic stem cell (HSC) sits atop the hierarchy of hematopoietic development and is characterized by its ability to both self-renew and give rise to all the different hematopoietic lineages for the lifetime of an organism (**Figure 1.1**). (Cumano and Godin, 2007; Orkin and Zon, 2008). While the concept of a hematopoietic stem cell had been proposed as early as the late 1800s (Ramalho-Santos and Willenbring, 2007), the first formal experimental evidence that supported the existence of an HSC is credited to Till and McCullouch who showed that transplantation of murine bone marrow cells into irradiated recipients led the formation of spleen colonies at a rate approximately proportional to the number of transplanted cells (McCulloch and Till, 1960; Till and McCulloch, 1961). This discovery initiated intense research into the genetic and molecular mechanisms that regulate the development and maintenance of the hematopoietic system, helping to make it one of the most well-characterized organs to date. The discovery of HSCs also revolutionized the treatment of many hematological malignancies including leukemia, lymphoma, and other hematopoietic disorders such as anemia through the use of bone marrow, and later, mobilized peripheral blood or cord blood transplantation therapy (Copelan, 2006).

Vertebrate Hematopoiesis

While the long-term repopulating HSC (LTR-HSC) is the ultimate generator of all the lineages in the blood system, it is a largely quiescent cell after it is established during

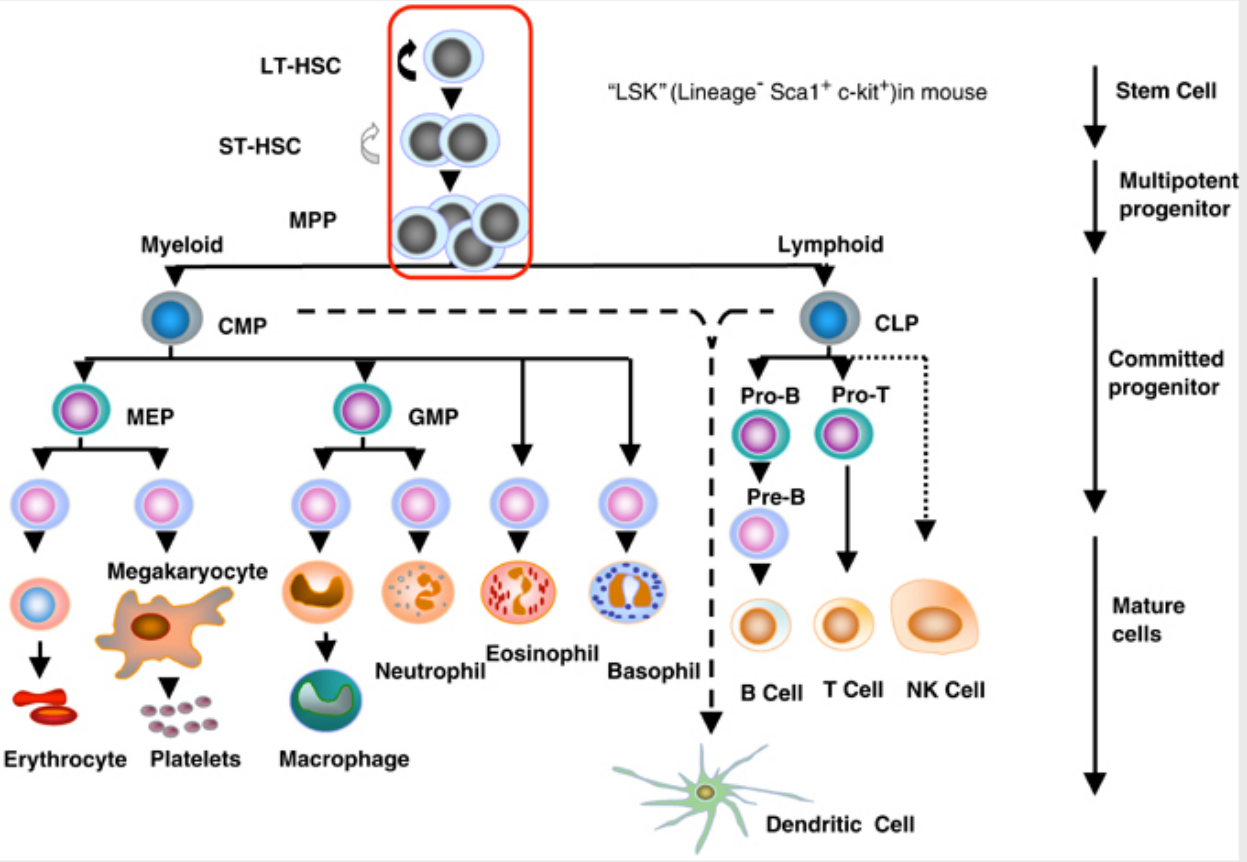


Figure 1.1

The hematopoietic stem cell (HSC) is responsible for the generation of all lineages of the blood system including lymphoid, myeloid, and erythroid cells. Reprinted by permission from Macmillan Publishers Ltd: Oncogene; (Larsson and Karlsson, 2005), copyright 2005.

development (Cheshier et al., 1999). Instead, the bulk of the cell divisions and proliferation in the hematopoietic system are performed by short-lived multipotent progenitors cells, sometimes termed short-term HSCs (ST-HSCs), and downstream committed precursor populations, such as Common Lymphoid and Myeloid Progenitor cells (Passegué et al., 2005). These progenitors give rise to the two major lineages of the hematopoietic system, erythro-myeloid and lymphoid (Wang and Wagers, 2011). The primary function of myeloid cells, including macrophages, neutrophils, and granulocytes, which make up the innate immune system, is to fight against infection in the host animal, while lymphoid derived cells (T and B cells) compose the adaptive immune system and are designed to recognize prior foreign pathogens to facilitate a quick biological response to secondary exposure (Iwasaki and Medzhitov, 2010; Trede et al., 2004).

Erythroid, or red blood cells, are hemoglobin carrying erythrocytes that are responsible for the transport of oxygen throughout the organism; defects in erythropoiesis give rise to many different forms of anemia (Dzierzak and Philipsen, 2013). The maturational cascade of erythrocytes is quite well understood and requires many different genes including *Scl* (Shivdasani et al., 1995), *Lmo2* (Warren et al., 1994), *Gata2* (Tsai et al., 1994) and *Gata1* (Fujiwara et al., 1996; Pevny et al., 1995; 1991); loss of any of these genes is embryonic lethal, underscoring their critical roles in early development. *Gata1* is considered a master regulator of erythrocyte maturation as it is required to for the activation of many erythroid genes (Ferreira et al., 2005; Welch et al., 2004). The combined transcriptional activation of these genes, along with other co-factors, ultimately interact to form a functional hemoglobin positive, oxygen-carrying erythrocyte, which is essential for embryonic development (McGrath and Palis, 2008).

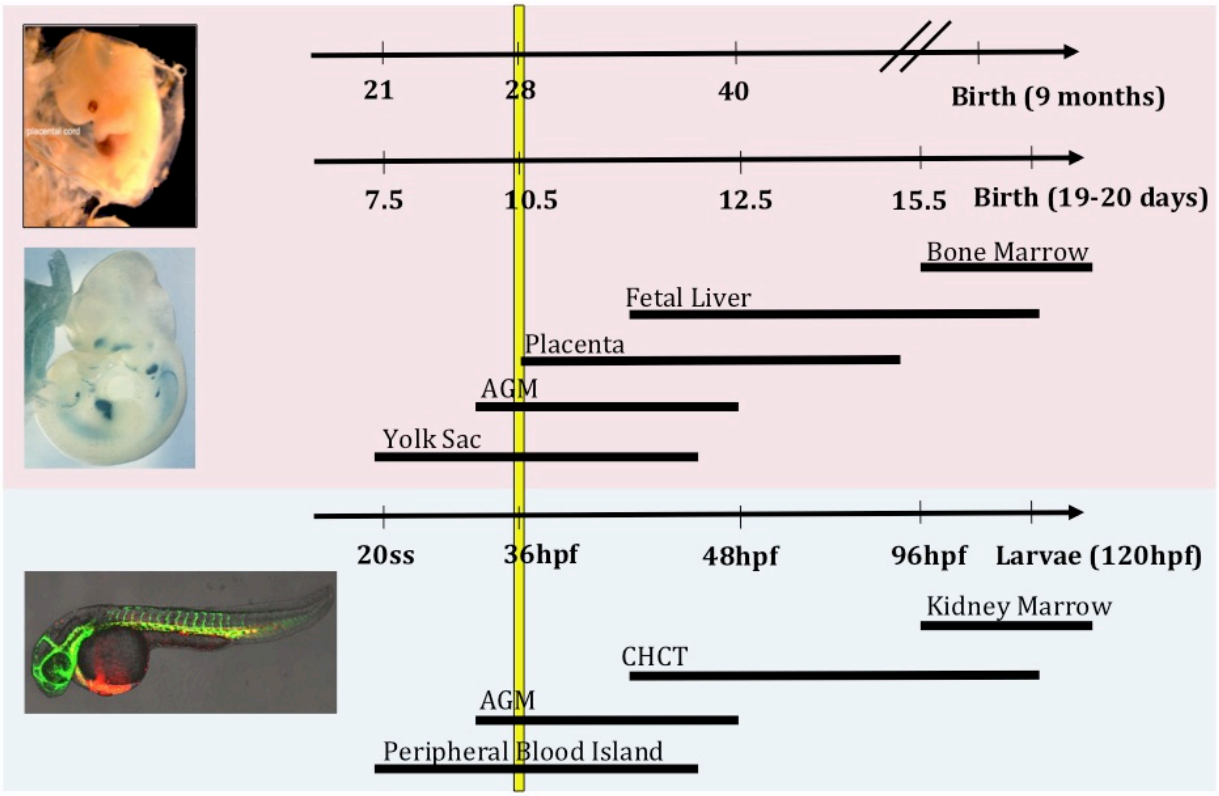


Figure 1.2 (Adapted from Trista North).
The sites of HSC development change throughout development. Across vertebrate species, HSCs are born in the AGM and subsequently migrate to other locations before reaching the bone marrow (human and mouse) or kidney marrow (zebrafish), the sites of hematopoiesis in adults.

The Developmental Biology of Murine HSCs

In contrast to the hierarchical differentiation from multipotent HSCs that occurs in the adult, during development, hematopoiesis initiates as a series of sequential waves, each with increasing competence (**Figure 1.2**). The first wave of hematopoiesis, termed primitive, gives rise to a transitory population of both erythroid and myeloid cells; these cells are largely formed in the extraembryonic yolk sac in mammals starting at embryonic day 7.5 (Dzierzak and Speck, 2008). During murine development, the first true hematopoietic stem cells emerge in the aorta-gonad-mesonephros (AGM) beginning at approximately embryonic day 10.5 (Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993; Sanchez et al., 1996); these cells are responsible for repopulating the blood system of the animal for its entire lifetime, making this a critical step during early embryonic development (Dzierzak and Speck, 2008; Medvinsky et al., 2011; Orkin and Zon, 2008). A similar process occurs during human gestation from roughly days 27 to 40 of gestation (Tavian and Peault, 2005). Interestingly, while the AGM is the location of the initial formation of HSCs, it only remains a hematopoietic site for a short period of time during development. Instead, the HSCs from the AGM migrate to the fetal liver at ~E14.5 in the mouse, where they are joined by a second wave of *de novo* produced HSCs derived slightly later in the placenta, as well the yolk sac; together, these cells continue to proliferate as well as differentiate to meet the needs of the growing fetus (Ema and Nakauchi, 2000; Mikkola and Orkin, 2006; Morrison et al., 1995; Müller et al., 1994). Finally, shortly after birth, HSCs egress from the fetal liver and travel to the bone marrow where they will remain in varying states of activity and quiescence for the lifetime of the organism, as many cell types in the bone marrow microenvironment (niche) work together to regulate HSC

maintenance and homeostasis (Christensen et al., 2004; Orkin and Zon, 2008; Zanjani et al., 1993). Interestingly, neither the fetal liver nor the bone marrow is thought to be responsible for the production of *de novo* HSCs, making the earliest steps in hematopoietic development in the AGM critical for the long-term viability of the organism (Dzierzak and Speck, 2008).

HSCs Arise from Hemogenic Endothelium in the AGM of Developing Vertebrates

It has been recognized since at least the 1920s that certain endothelial cells in the blood vessels of developing embryos appeared "hemogenic" (Maximow, 1924). However, it was unclear if HSCs were generated *de novo* in the AGM or if they arose as a result of migration from another spatial location in the developing embryo. Early work utilized chick-quail chimeric grafts to demonstrate that definitive hematopoietic stem cells arise from an intraembryonic location, rather than from the extraembryonic yolk sac, which was the accepted source of definitive HSCs at the time (Dieterlen-Lievre, 1975). The intraembryonic generation of HSCs was later confirmed using explant cultures of dissected AGMs. This work revealed that HSCs in the AGM are generated autonomously, as HSCs were formed in the absence of both blood flow and other potential cellular sources (Medvinsky and Dzierzak, 1996). More recent work has focused on elucidating the cells within the AGM that are responsible for the generation of HSCs. The related ontogeny of the first definitive HSCs and the vessels is supported by multiple pieces of evidence including the closely related spatial emergence of budding clusters of HSCs in the wall of aortic specified endothelium as well as a high degree of expression of common genetic markers and regulators (Cumano and Godin, 2007).

Recent studies have largely indicated that HSCs arise from the endothelial cells lining the ventral wall of the dorsal aorta. The emergence of HSCs from the wall of the AGM, the start of a process whereby newly produced HSCs bud from the endothelium, enter into the circulation of the embryo and travel to seed the next site of hematopoiesis, was termed "endothelial to hematopoietic transition (EHT)" for its similarities to extravasation in cancer (Kissa and Herbomel, 2010). EHT appears to be highly conserved across vertebrates, as similar movements were noted in both murine (Boisset et al., 2010) and zebrafish systems (Bertrand et al., 2010; Kissa and Herbomel, 2010). Though it is now well-established that HSCs are born from hemogenic endothelial cells in the wall of the dorsal aorta, the signals that control the spatial localization of HSCs to the ventral wall of the aorta are not well elucidated, nor are those that initiate the onset of this process.

Runx1 is a Critical Regulator of HSC Development

While many genes play important roles in the development of the hematopoietic system, one of the best characterized is *Runx1*, also known as *AML1/Cbfa2*. *Runx1* knockout mice die at ~E12.5 due to hemorrhaging in the central nervous system; they also displayed deficits in fetal liver hematopoiesis (Wang et al., 1996). Later studies revealed that *Runx1* is expressed in both HSCs and the endothelial cells from which HSCs emerge at E10.5 (**Figure 1.3**); *Runx1* knockout mice were also shown to lack AGM HSCs at E10.5, suggesting that their fetal liver hematopoiesis defects result from a lack of definitive hematopoiesis in the AGM rather than a failure of the HSCs to home to the fetal liver (North et al., 1999). Prospective isolation of *Runx1* positive cells was also shown to enrich for adult

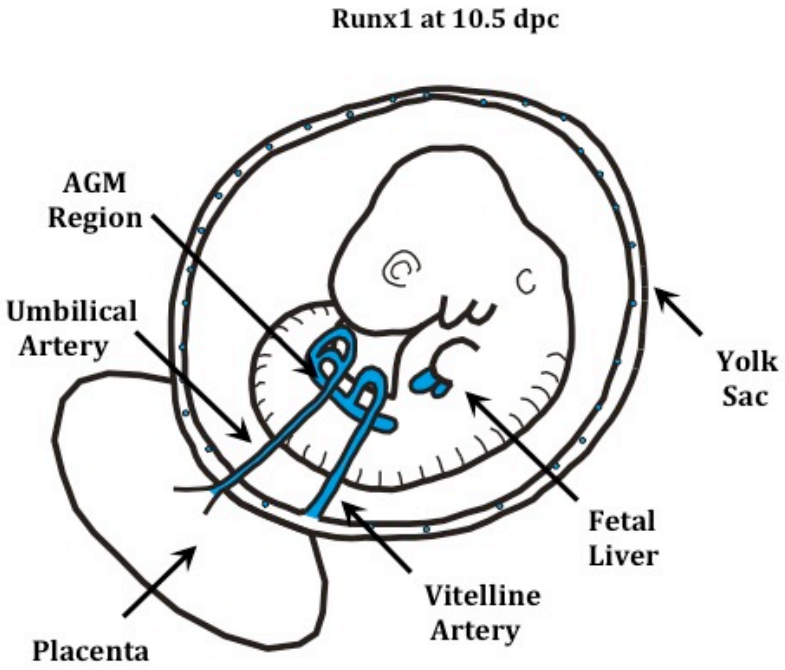


Figure 1.3 (Adapted from Trista North).
At E10.5, *Runx1* (as indicated by blue) is expressed in endothelial cells in the AGM and Umbilical and Vitelline Arteries as well as in HSCs.

repopulating activity after transplant, confirming that the expression of *Runx1* does indeed mark the first definitive HSCs (North et al., 2002). More recent work has demonstrated that *Runx1* is required for the endothelial to hematopoietic transition that occurs during the budding of HSCs from the endothelium, but not after (Chen et al., 2009), helping to provide temporal information on the role of *Runx1* in hematopoietic development. While *Runx1* is largely recognized for its seminal role in HSC specification, it is also commonly mutated in hematological malignancies, suggesting that it continues to play a role in hematopoietic homeostasis, even after the development of AGM HSCs (Cohen, 2009). Consistent with this

idea, HSCs in the adult express *Runx1*, as do lymphoid and myeloid subtypes, indicating it remains an important regulator of hematopoiesis, although its precise role in the adult has not been fully characterized (Lorsbach et al., 2004; North et al., 2004).

Zebrafish as a Model System for Developmental Biology

While the zebrafish (*Danio rerio*) was first suggested for use in hematology research in 1963 (Colle-Vandevelde, 1963), it is only in the last ~20 years that it has truly risen to prominence as one of the preeminent systems for hematopoietic stem cell (HSC) biology, particularly in the areas of development and regeneration. The zebrafish has emerged as a highly tractable model system for scientific research due in large part to the external fertilization of embryos and their optically clear development, allowing for real-time *in vivo* observation of developmental processes. Additionally, the ability of fecund females to lay hundreds of embryos per week enables rapid high-throughput experimentation and strong statistical analysis of phenotypes. Zebrafish are particularly useful for hematology research due to the high conservation of genetic factors regulating blood development as well as the structure and function of hematopoietic cell types, and the ability to visualize circulating erythrocytes with only a dissecting microscope.

Hematopoiesis is Highly Conserved in the Zebrafish Model

As in all other vertebrates analyzed to date, zebrafish hematopoiesis occurs in multiple phases (**Figure 1.4 and Figure 1.5**). Primitive hematopoiesis, the first wave, occurs from ~12 to 24 hours post fertilization (hpf) in two anatomically distinct locations: a section of posterior lateral mesoderm called the inner cell mass gives rise primarily to

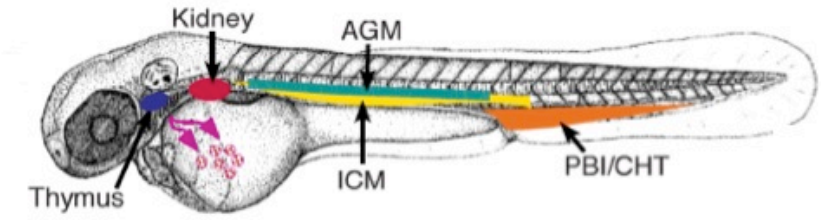


Figure 1.4 (Adapted from Trista North and David Traver).
Primitive erythropoiesis in the zebrafish occurs in the Inner Cell Mass (ICM). The first definitive HSCs are born in the Aorta-Gonad-Mesonephros (AGM) before migrating to the Caudal Hematopoietic Tissue (CHT), followed by the thymus and kidney marrow, the sites of hematopoiesis in the adult.

cells of erythroid lineage (Detrich et al., 1995), while the rostral blood island in the anterior portion of the embryo gives rise to a primitive macrophage population (Herbomel et al., 1999; Lieschke et al., 2002). The process of erythropoiesis requires many of the same genes that are utilized during primitive hematopoiesis in other vertebrate species including *scl* (Dooley et al., 2005), *gata1* (Lyons et al., 2002), *lmo2* (Patterson et al., 2007), and *tif1* (Ransom et al., 2004) while the generation of myeloid cells requires *pu.1* and *cebpα* (Hsu et al., 2004; Lyons et al., 2001; Su et al., 2007). A transient wave of definitive hematopoietic progenitors has also been recently identified, termed erythromyeloid precursors (EMPs), that are present in the embryo prior to the emergence of true multi-lineage HSCs (Bertrand et al., 2007a). These progenitors give rise to both definitive erythroid and myeloid (neutrophilic granulocytes, monocytes, and macrophage) colonies in culture. However, EMPs were never observed to populate the kidney marrow or thymus *in vivo*, indicating a

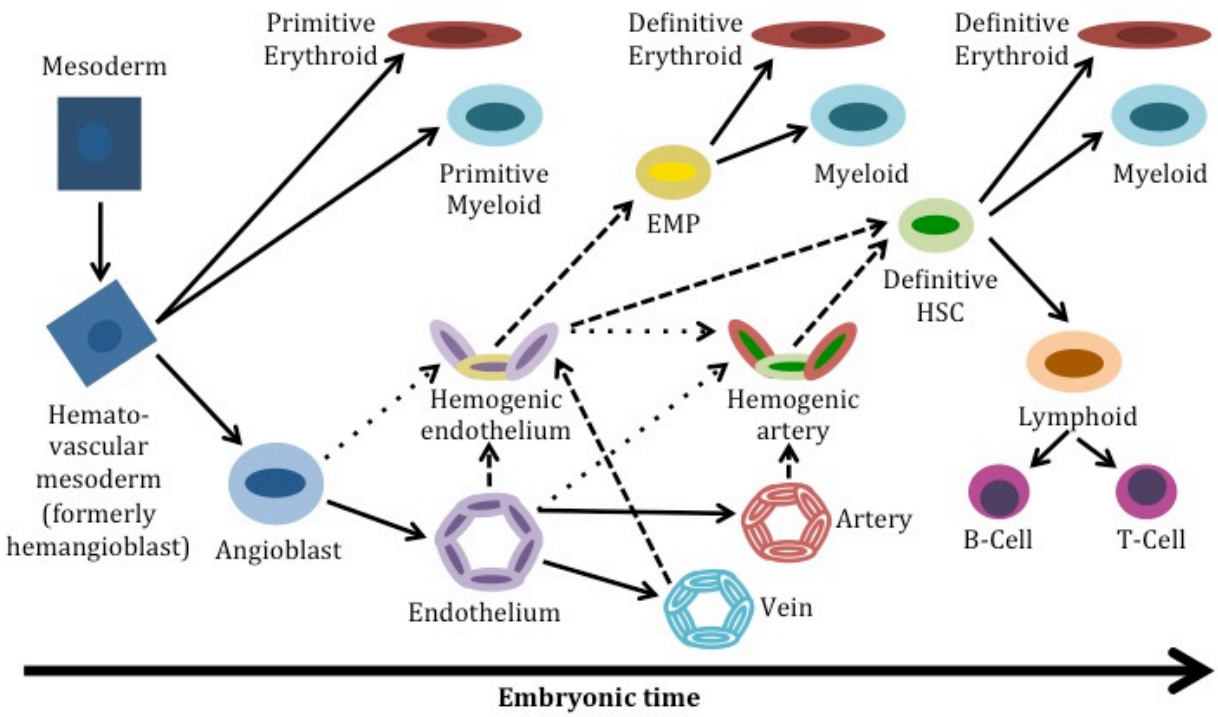


Figure 1.5:
Schematic representation of embryonic hematopoietic development showing lineage relationships between the hemato-vascular populations. Solid lines indicate known lineage choices while dotted lines indicate putative decisions.

lack of lymphoid potential and supporting their classification as a hematopoietic progenitor rather than a true HSC. Definitive HSCs arise from hemogenic endothelium in the ventral wall of the dorsal aorta in the Aorta-Gonad-Mesonephros (AGM) beginning at ~30 hpf (Bertrand et al., 2010; Kissa and Herbomel, 2010) shortly after the onset of circulation (North et al., 2009), a process which is highly conserved among vertebrate species. Following their emergence in the AGM, HSCs migrate to the Caudal Hematopoietic Tissue (CHT), thought to be the maturational equivalent of the mammalian fetal liver, where they expand in number and subsequently seed the thymus and kidney marrow, the primary sites of adult hematopoiesis in the fish (Murayama et al., 2006).

Many of the genetic factors that specify the emergence of definitive HSCs are well-elucidated, and are largely consistent with those required in mammalian systems, indicating a high degree of evolutionary conservation. A common cascade of transcription factors including sonic hedgehog, followed by Vascular Endothelial Growth Factor (VEGF) (Gering and Patient, 2005; Lawson et al., 2002), and Notch (Burns, 2005; Lawson et al., 2001) regulates the development of both arterial-specified endothelium as well as HSCs, reflecting the spatial emergence of HSCs from the ventral endothelial wall of the dorsal aorta in the developing embryo (Bertrand et al., 2010; Kissa and Herbomel, 2010). These factors lie upstream of both *scl* (Kim et al., 2013) and the transcription factor *runx1*, also a key regulator of HSC development, in the fish (Kalev-Zylinska et al., 2002). Not only are the genetic factors regulating HSC emergence conserved across species, the function of the different blood lineages appears to be highly conserved as well. Although zebrafish erythrocytes remain nucleated throughout their lifespan, they express the same globin genes that are found in mammals (Ganis et al., 2012), indicative of a similar function. Zebrafish also contain thrombocytes (platelets) (Lin, 2005) that, as in mammals, play a role in blood clotting (Jagadeeswaran et al., 1999). In addition, the cellular components of the innate immune system are also highly conserved; zebrafish contain granulocytes (Lieschke et al., 2001) as well as macrophages (Herbomel et al., 1999) and neutrophils (Bennett et al., 2001) in the myeloid lineage. Migration of *myeloperoxidase+* granulocytes toward sites of injury and inflammation was observed *in vivo*, confirming that these cells maintain their anti-inflammatory properties in the zebrafish (Lieschke et al., 2001); similarly, macrophages maintain their ability to phagocytose apoptotic cells and bacteria (Herbomel et al., 1999). Zebrafish also appear to possess a full component of cells of the adaptive

immune system. *Recombination Activating Gene* (*rag*) positive T-lymphocytes are found in the thymus as early as 3.5 dpf by *in situ* hybridization (Keightley et al., 2011); B-cells have similarly been reported, although their site of maturation remains controversial (Danilova and Steiner, 2002; Page et al., 2013) and Natural Killer cells are also thought to exist (Yoder et al., 2001; 2004). In sum, the high conservation of both genetics and function of the hematopoietic cells in zebrafish lends validity to their use in hematology research.

Genetic Manipulation in the Zebrafish Model

Beyond the strong conservation of factors regulating HSC development in the zebrafish, the system is also highly amenable to genetic manipulation, making it useful for identification of novel regulatory factors, as well as gain and loss-of-function studies. While homologous recombination akin to murine models remains difficult to achieve, knockdown of a given gene(s) in the zebrafish system is readily performed using morpholino technology. Morpholinos (MOs) are modified antisense oligonucleotides designed to block either splicing or translation of a specific mRNA, thereby resulting in a knockdown of function of the target gene due to a lack of translation. MOs are microinjected into zebrafish embryos at the 1-cell stage and they remain effective for the first ~5 days of development (depending on the dose) (Nasevicius and Ekker, 2000), making the technology critical for its unique ability to rapidly assess the effects of loss of a given gene on vertebrate hematopoietic development. Other significant benefits of the MO system include the ability to titrate the dose of MO injected, making it possible to carefully control the degree of knockdown, often allowing one to bypass embryonic lethal mutations, as well the ease with which suppression of multiple genes can be examined at once via combinatorial injection.

However, there are caveats to the use of MOs, including potential toxicity of the MO and/or injection procedure, off-target effects, and the inability to look at the consequences of long-term loss of function of a given gene(s). More recent technologies, including zinc finger nucleases, TALENS and the CRISPR/CAS system, have emerged as promising techniques to make specific, germline-transmissible mutations of genes of interest (Cade et al., 2012; Hwang et al., 2013; Meng et al., 2008; Sander et al., 2011). These techniques make it possible to generate full knockouts (null phenotypes) in a fairly timely manner and will enable better assessment of phenotypes that emerge due to loss of a gene after MO technology is no longer useful, such as in adult or cancer studies.

The previously discussed benefits of the zebrafish system including external transparent development and large clutch size have historically enabled the isolation of many hematopoietic mutant lines through large-scale mutagenesis screens. Significantly, zebrafish embryos are able to survive for at least 10 days in the absence of blood flow and heartbeat, enabling identification and analysis of mutations that would be impossible to study in mammalian systems due to early embryonic lethality (Rombough and Drader, 2009; Stainier et al., 1996); additionally, as the liver is not a site of embryonic hematopoiesis (Murayama et al., 2006), mutations affecting the gastro-intestinal system will not generally impair hematopoietic development. One of the most common methods to introduce mutations into the germline of zebrafish involves the use of N-ethyl N-nitrourea (ENU) (Driever et al., 1996; Ransom et al., 1997; Weinstein et al., 1996), a mutagen which results in the induction of point mutations. The majority of the hematopoietic mutants isolated from the first ENU screens involved deficits in erythrocyte function. These mutants included well known master erythrocyte regulators, including *gata1* in the *vlad tepes* line

(Lyons et al., 2002) and *globin* in *zinfandel* zebrafish (Brownlie et al., 2003), confirming the high conservation of hematopoietic regulation across species. More importantly, however, some of the mutations led to the identification of novel factors involved in erythrocyte maturation and function such as ferroportin (Donovan et al., 2000) and mitoferrin (Shaw et al., 2006); mutations in the *ferroportin* gene have subsequently been identified as one of the most common causes of iron overload in humans (Pietrangelo, 2004), making it a prime example of the utility of zebrafish screening for the identification of causal genes relevant to human disease. The zebrafish is also an excellent model for several different forms of anemia, including Hereditary Elliptocytosis, Microcytic Anemia, and Congenital Dyserythropoietic Anemia, making them potentially useful for the identification of compounds or genes that alter the severity of anemic phenotypes (Donovan et al., 2002; Paw et al., 2003; Shafizadeh et al., 2002).

Interestingly, zebrafish embryos can survive for several days as a haploid organism. As a result, UV inactivated sperm can be used to fertilize the eggs of females from the F1 generation of an ENU screen, thereby resulting in the formation of haploid embryos, of which 50% are recessive for a mutated gene and allowing assessment of phenotypes one generation prior to what would normally be possible in traditional zebrafish or mammalian screens (Walker, 1999). This technique has revealed genes that are important in hematopoiesis including T-cell development (Trede et al., 2008) and vasculogenesis, a process intimately tied to HSC specification and emergence (Covassin et al., 2009). Another common method of inducing mutations in the zebrafish genome involves retroviral insertional mutagenesis (Amsterdam et al., 1999; Gaiano et al., 1996), whereby retroviral DNA is randomly integrated into the genome, resulting in disruption of gene expression

and/or function. Analysis of zebrafish mutants from one such insertional mutagenesis screened aided in the identification and sequential organization of a hierarchy of factors required for HSC emergence in the developing vertebrate embryo including *VEGFAa, Notch, HDAC, and Runx1* (Burns et al., 2009).

As discussed above, one of the most critical genes in the specification of HSCs during development is *runx1*. When *runx1* was knocked out, mutant embryos failed to undergo AGM definitive hematopoiesis and showed an absence of definitive lineages at 5 days post-fertilization (Sood et al., 2010). While most null animals died between days 11 and 20 post-fertilization, a small fraction survived until adulthood. The reasons for this are not well known, but could be due to the presence of normal erythromyeloid progenitors that fulfill some of the role of HSCs enabling some of the null animals to survive. In addition to *runx1*, the gene *cmyb* is recognized as a conserved HSC marker (Mucenski et al., 1991; North et al., 2007). *cmyb* null fish show an absence of definitive hematopoiesis but are able to survive into adulthood, likely because of the ability of oxygen to diffuse into the fish from the water, making the requirement for oxygen-carrying erythrocyte capacity less critical (Soza-Ried et al., 2010). In contrast, the *cmyb* knockout mouse dies at E15 (Mucenski et al., 1991), making the zebrafish knockout particularly useful for analyzing effects of *cmyb* on hematopoiesis during later developmental stages.

One of the most enigmatic mutants isolated in the large-scale ENU screens of the 1990s was *cloche* (Stainier et al., 1995). These embryos show broad hematovascular defects, with a strong reduction in the formation of both the blood and vasculature, as well as heart defects. The *cloche* zebrafish are considered to be one of the strongest pieces of evidence for the "hemangioblast", a theoretical bipotential cell population that supports

hematopoietic and vascular development, as a single mutation produces both vessel and hematopoietic defects. While many attempts to clone the *cloche* gene have been made (Weber et al., 2005; Xiong et al., 2008), the identity of the exact genetic mutation underlying the *cloche* phenotype remains unknown, as loss of a single gene has been unable to replicate all the phenotypes observed in the *cloche* mutant. Beyond its location in a telomeric region, the difficulty of cloning *cloche*, which is inherited in a recessive fashion (Stainier et al., 1995), raises the intriguing possibility that the mutation may lie in an intergenic or non-coding region, such as a miRNA or lncRNA that has the ability to impact the transcription or translation of multiple genes. While simply speculation, a mutation in one of these pleiotropic regulatory regions may explain why the *cloche* phenotype is so penetrant, leading to defects in multiple aspects of hemato-vascular development.

Zebrafish were also recently used for a MO-based "knockdown" screen to identify epigenetic factors with specific regulatory roles in hematopoiesis. This relatively high-throughput reverse-genetics screen resulted in the identification of several families of epigenetic regulators that impact either primitive or definitive hematopoiesis, or both, and provides insight into the role of epigenetic regulation in establishing hematopoietic commitment and expansion (Huang et al., 2013). Zebrafish were also used in a genetic modifier screen to look for suppressors of the *moonshine* mutant, which has defects in erythropoiesis; this screen resulted in the identification of transcriptional elongation as a regulator of cell fate (Bai et al., 2010).

Real-time Imaging of Hematopoietic Stem Cell Emergence During Development

The strong genetic conservation in zebrafish has also enabled engineering of many transgenic reporter lines that express fluorescent proteins under the control of promoter regions of given hematopoietic genes of interest. These transgenic lines have been especially useful for quantifying alterations in hematopoietic cell number, particularly given the lack of cross-reactivity with many existing mammalian antibodies, and for imaging the emergence and migration of HSCs during development *in vivo* in real time, a process which is extremely difficult in mammalian systems (Bertrand et al., 2010; Kalev-Zylinska et al., 2002; Kissa and Herbomel, 2010). While many primitive hematopoietic and vascular reporter lines emerged in the late 1990s and early 2000s including *gata1* (Long et al., 1997), *lmo2* (Zhu et al., 2005), and *fli1a* (Lawson and Weinstein, 2002), the earliest transgenic HSC-reporter line to emerge was the *Tg(-6.0itga2b (CD41):EGFP)* line, which marks mature HSCs and is commonly used to visualize HSCs after their emergence from the AGM (Lin, 2005; Ma et al., 2011), particularly in the CHT and kidney marrow. Importantly, while also expressed at high levels on thrombocytes, the utility of CD41 as a blood stem cell marker appears to be conserved as it has also been reported to be present on HSCs during mammalian development (Mikkola et al., 2003).

Over the past several years, elegant time-lapse imaging studies have enabled real-time, *in vivo* visualization of HSC emergence from hemogenic endothelium in the AGM. Co-expression of *cmyb,* another conserved HSC marker (Mucenski et al., 1991; North et al., 2007) and *flk1* transgenes was shown by lineage tracing to mark budding HSCs in the vasculature (Bertrand et al., 2010) and has been used to enable precise quantification and visualization of HSCs in the embryo. Simultaneously, time-lapse imaging of embryos

expressing GFP under the control of the *flk1* transgene was utilized to visualize the process of budding and egress of HSCs from the endothelium into the vasculature, now termed endothelial-to-hematopoietic transition (EHT) (Kissa and Herbomel, 2010). These studies both lent strong support to the theory that HSCs arise from a subset of endothelial cells, termed hemogenic endothelium, found in the vasculature, and the dorsal aorta in particular. This argument was further bolstered by visualization of *runx1* transgenic reporter fish which indicated that *runx1* was expressed in the AGM and in particular in the ventral wall of the dorsal aorta, consistent with its function in hemogenic endothelium in mammalian models (Chen et al., 2009; Lam et al., 2010; North et al., 1999; Yi Ni Lam et al., 2009). The role of *runx1* in EHT in zebrafish was functionally demonstrated *in vivo* via MO injection, as *runx1* knockdown resulted in a lower rate of budding initiation, as well as cell abortion upon attempting to exit the aortic wall (Kissa and Herbomel, 2010), a finding consistent with observations from murine models (Chen et al., 2009). Interestingly, both zebrafish imaging studies indicated that HSCs, upon leaving the endothelium, enter into circulation via the vein rather than the artery, a process that appears to be unique in the fish (Bertrand et al., 2010; Kissa and Herbomel, 2010); the explanation for this difference could potentially result from the overlapping rather than continuous structure of the venous endothelium that enables easier intravasation of cells (Kissa et al., 2008) or may simply reflect the compact spatial alignment of the major trunk vessels in zebrafish compared with that of mammalian embryos, allowing local chemoattractants from the vein to reach newly produced HSCs immediately upon egress rather than in the circulation. In addition to *in vivo* documentation of the emergence of HSCs performed in the zebrafish system, concurrent studies using explant cultures have indicated a very similar process

occurs in the mouse (Boisset et al., 2010), suggesting that this series of events is highly conserved. Transgenic HSC reporter lines can also be used to visualize activity in secondary sites of hematopoiesis including the CHT, thymus, and kidney, enabling their use for lineage tracing as well as analyzing migration, colonization and expansion of HSCs (Lam et al., 2010; Yi Ni Lam et al., 2009). In addition to further genetic profiling of labeled populations (Weber et al., 2005), our recent study has also indicated that these transgenic lines (for example CD41) can be utilized to enrich for embryonic HSCs with adult kidney marrow repopulating activity by FACS sorting and transplantation (Harris et al., 2013). Together, these studies confirmed the *in vivo* functional potential of the labeled populations of interest and provide an additional resource for the prospective isolation and analysis of factors regulating HSCs during vertebrate development.

In Vivo High-Throughput Small Molecule Screening in the Zebrafish

Due the to the large numbers of embryos available from pair-wise mating(s) and the external development of the fish, it is relatively easy to do large-scale chemical screening for small molecule modifiers in zebrafish by simple addition of "test" compounds to the fish water. The first chemical screen was published in 2004 and was aimed at the identification of novel suppressors of the *gridlock* (*grl*) phenotype, caused by a mutation in *hey2*, that leads to aortic coarction and defects in arterial/venous development (Peterson et al., 2004). The compound discovered, GS4012, was a potent inducer of VEGF and was the first "therapeutic" discovery in zebrafish, resulting in a reduction of the severity of a genetic disorder impacting the hematovascular system.

The first hematopoietic screen used the *Tg(gata1:GFP)* line to identify modifiers of primitive hematopoiesis (Shafizadeh et al., 2004); subsequent screens have revealed other novel modifiers of both primitive and definitive hematopoiesis (North et al., 2007; 2009; Paik et al., 2010). One screen hit that has shown direct therapeutic potential was ProstaglandinE2, which robustly increased the number of *runx1+* HSCs in zebrafish and mice (North et al., 2007), in part through modulation of the Wnt pathway (Goessling et al., 2009); following translational work (Goessling et al., 2011), PGE2 became the first compound derived from drug discovery in the zebrafish to reach FDA-approved clinical trial testing in human patients (Cutler et al., 2013). Other significant discoveries made via the chemical screening approach yielded compounds which could induce hematopoietic differentiation in the AML-ETO model of leukemia (Yeh et al., 2007; 2009). A subsequent screen also identified compounds that were specifically toxic to immature T-cells, such as Lenaldeker, a potential chemotherapeutic agent for patients with T-cell Acute Lymphoblastic Leukemia (T-ALL) (Ridges et al., 2012). While *in vitro* screening of cell populations of interest is certainly a valuable methodology for therapeutic compound identification, *in vivo* screening using the zebrafish model has the advantage of identifying drug toxicity "side effects" from the outset, as well as allowing for production and evaluation of any compound metabolites that may be produced, boosting the translational potential of screening hits and speeding the time from discovery to application.

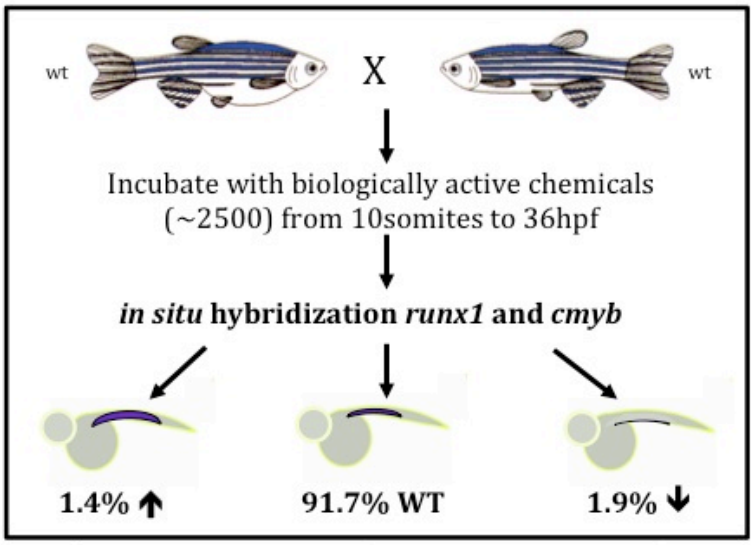
Adult Hematopoietic Assays Adapted for Use in the Zebrafish Model

While the bulk of the hematopoietic studies done in zebrafish have been performed using embryos, a growing number of techniques to study HSC biology in the adult animal

are emerging. One of the primary challenges of working with zebrafish to study hematopoiesis in the adult is the lack of antibodies for cell surface markers to enable FACS analysis of HSCs and the different hematopoietic lineages. The first study to identify a simple way to isolate whole kidney marrow (WKM; the site of adult hematopoiesis) and analyze the cellular content of the marrow relied upon flow cytometry using cell size (forward scatter) and granularity (side scatter) to isolate the various lineage fractions (Traver et al., 2003); while a similar technique has also been used in the mouse (Dzierzak and de Bruijn, 2002), this was a huge advancement for studying hematopoietic cell production and homeostasis in zebrafish. Subsequent studies advanced the functional utility of the WKM dissection and analysis by introducing irradiation-mediated ablation of the hematopoietic system to both enable assessment of KM recovery after injury and to perform adult-to-adult HSC transplantation (Traver et al., 2004), long considered the best technique to determine the presence of a true multipotent hematopoietic stem cell. Transplantation methodology was further enhanced by the generation of *casper* fish, which lack pigmentation, enabling real-time *in vivo* visualization of HSC homing and engraftment. Transplantation of *beta-actin:GFP* WKM donor cells in irradiated *casper* recipients enabled imaging of the donor cells from 2 hours until at least 5 weeks after transplantation (White et al., 2008). This technique has significant advantages over most analyses of homing in mammalian systems where visualization of the process in real-time is difficult due to the deep interior location of the bone marrow niche. Other studies focused on the role of the Major Histocompatibility Complex (MHC) and indicated that matching the MHC type between donor and recipient drastically improved rates of engraftment (de Jong et al., 2011). Later efforts took advantage of the ability to study hematopoietic ablation and

recovery by identifying several factors, including Notch signaling and PGE2/Wnt, which could accelerate the hematopoietic recovery of the fish, providing potential clinically useful insights into the engraftment of cells following bone marrow or cord blood transplantation and strengthening the connection between regulators of embryonic development and tissue regeneration (Burns, 2005; Goessling et al., 2009). Importantly, while PGE2 was initially identified as a modifier of HSC regeneration in the zebrafish, it was simultaneously found to play a similar role in the mouse (North et al., 2007), emphasizing the utility of the zebrafish to identify critical pathways in HSC regulation across vertebrates. Though zebrafish cells in general have traditionally been difficult to maintain *in vitro*, recent studies indicate that long-term hematopoietic culture of isolated kidney marrow stem and progenitor cells is possible, as both erythroid and myeloid colonies were generated in a clonal methylcellulose assay (Stachura et al., 2011) following identification and isolation of zebrafish specific cytokines such as Erythropoietin (Epo) and Granulocyte Stimulating Colony Factor (GCSF). This advancement opens further avenues of functional analysis and manipulation of hematopoietic stem and progenitor cell biology in the zebrafish. Finally, while not yet examined in detail, the localization of the HSC niche within the kidney marrow rather than bone marrow niche, may itself prove advantageous to elucidation of cell autonomous and/or bone independent regulatory effects on HSCs and hematopoietic progenitors.

One of the most prominent areas of zebrafish hematology research is currently in cancer biology, particularly that of T-ALL, which was the first model of cancer in the fish (Langenau et al., 2003) and is the most frequent childhood hematological malignancy (Kaatsch, 2010). While the zebrafish has not been a long-standing cancer model, it is



Compound	Classification
Chlorotrianisene	Synthetic Estrogen
Chrysin	Flavone, Aromatase Inhibitor
17b-estradiol	Estrogen
Flurandrenolide	Synthetic Steroid
Formonetin	Isoflavone, Phytoestrogen
Norethindrone	Synthetic Progestin

Figure 1.6

A chemical screen led to the identification of estrogen and estrogen-related molecules as novel modifiers of the expression of *runx1* and *cmyb* in the zebrafish AGM.

quickly gaining traction due to the ease of chemical and genetic screening for modifiers of cancer development or progression (Langenau et al., 2003; 2005). Recent studies have led to the identification of the COX/β-catenin (Yeh et al., 2009; Zhang et al., 2013), PTEN (Gutierrez et al., 2011), S1P1 and ICAM1 pathways (Feng et al., 2010) as playing key roles in cancer development and progression using zebrafish models of hematologic malignancy. Screening for chemical modifiers of T-ALL progression also led to the identification of perphenazine, an antipsychotic, as an inducer of apoptosis in malignant cells; importantly, this was true in zebrafish, mouse, and human T-ALL cells (Gutierrez et al., 2014), indicating the usefulness of the fish for identifying drugs that can impact the course of human cancer.

Identification of Estrogen as an HSC Modifier

As described above, one of the benefits of the zebrafish as a model system is the ease with which large-scale chemical screens can be performed. The chemical screen which led to the identification of ProstaglandinE2 (PGE2) as a potent enhancer of HSC number (North et al., 2007) also identified multiple other signaling pathways or physiological cues that altered the development of HSCs including blood flow/Nitric Oxide (North et al., 2009) and metabolism/hypoxia (Harris et al., 2013). Another class of compounds identified in that screen was estrogen and estrogen related molecules (**Figure 1.6**), the focus of this thesis.

Estrogen is a Critical Steroid Hormone

Estrogen is a highly conserved steroid hormone. While it is ultimately a derivative of cholesterol, the most proximal step in its production is catalyzed by the enzyme aromatase (CYP19A) from the precursor testosterone. 17β-estradiol, commonly referred to as

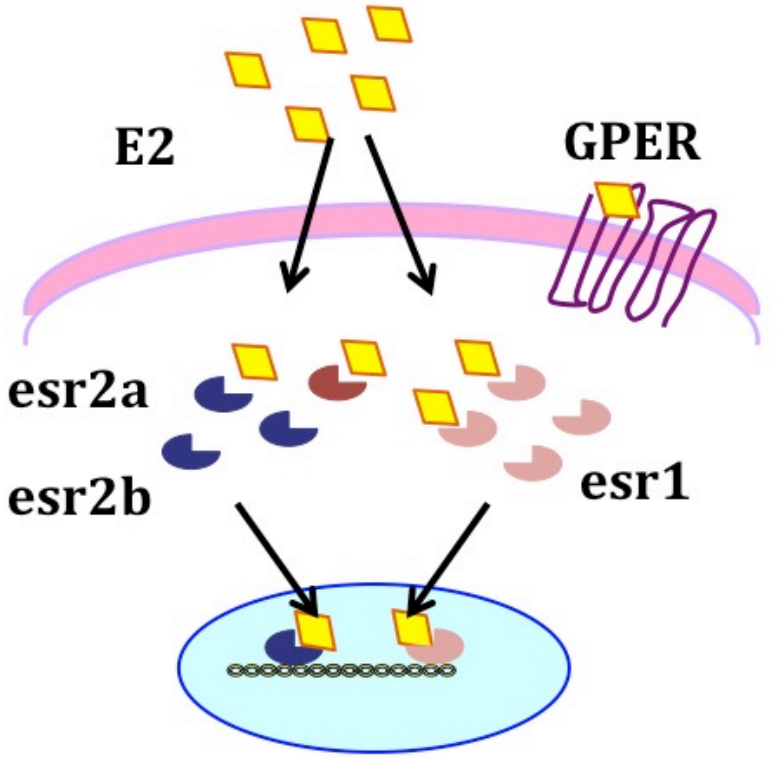


Figure 1.7

Estrogen can bind to the transmembrane G-Protein coupled receptor (GPER) or can freely diffuse into the cell. Once in the cell, estrogen binds to the nuclear hormone receptors (Esr1, Esr2a, and Esr2b) before translocating into the nucleus to bind to estrogen response elements (ERE).

estrogen (E2), is the most potent member of the estrogen family. However, other estrogen derivatives, such as estrone and estriol, are endogenously found in the vertebrate body (Heldring et al., 2007; Simpson and Davis, 2001; Tulchinsky et al., 1972). Estrogen has long been known to bind to one of two cytoplasmic receptors, Esr1 (ERα) or Esr2 (ERβ) (**Figure 1.7**), both members of the nuclear hormone receptor superfamily, to exert the majority of its effects (Heldring et al., 2007). Members of the nuclear hormone receptor family generally have similar physical structures, including a variable NH2 terminus, a DNA

binding domain, linker domains, and a ligand binding domain. As a result of the high homology in their structures, most nuclear hormone receptor family members have similar functions and all bind to hormone response elements that are found in the promoters of genes, leading to the classification of nuclear hormone receptors as transcription factors (Aranda and Pascual, 2001).

After the estrogen ligand binds to its receptor, the complex translocates into the nucleus where it binds to Estrogen Response Elements (consensus sequence GGTCAnnnTGACC) that are found in the promoter region of estrogen responsive genes and acts as a transcription factor complex (Björnström and Sjöberg, 2005; Klinge, 2001). While the majority of estrogen's currently characterized effects appear to be mediated by the nuclear hormone (cytoplasmic) estrogen receptors, a more recently discovered G-protein coupled receptor has also been identified, indicating that estrogen has the ability to mediate rapid, non-genomic signaling in both the mouse (Revankar et al., 2005) and in zebrafish (Liu et al., 2009). As a result of a partial genome duplication in evolutionary history, the zebrafish contains 3 cytoplasmic nuclear hormone receptors (Esr1, Esr2a, and Esr2b) (Menuet et al., 2002).

Estrogen and Blood Regulation

Although the role of estrogen in sexual development and reproduction has long been appreciated (Wilson and Davies, 2007), less is known about a putative role for estrogen in the hematopoietic system. However, some evidence suggests that estrogen does have the ability to modulate hematopoietic homeostasis, particularly in the context of hematologic malignancies. Mice with homozygous deletion of *Esr2* showed a predisposition

for the development of myeloproliferative disorders in the latter part of their lifespan (Shim et al., 2003) while mice that lacked the enzyme aromatase showed increased B lymphopoiesis and developed a lymphoproliferative autoimmune disorder (Shim et al., 2004), indicating that estrogen may play a role in the homeostasis of the blood system during adulthood. In addition, a splice variant of *Esr2* was elevated in chronic lymphocytic leukemia (Yakimchuk et al., 2012) and treatment with Esr2 agonists has been suggested as a putative chemotherapeutic for hematopoietic malignancies, further supporting a role for the estrogen system in hematopoiesis during adulthood (Yakimchuk et al., 2011). Estrogen is also known to play a role lymphopoiesis (Medina and Kincade, 1994; Medina et al., 1993), confirming it has the ability to impact at least some aspects of hematopoietic differentiation outside the context of disease.

The addition of exogenous estrogen has been reported to have opposing effects on the number and function of HSCs in the bone marrow in adult animals. While one group reported that excess E2 enhanced the number of HSCs found in the marrow (Illing et al., 2012) another found the opposite, saying that exposure to E2 decreased the number of HSCs in the bone marrow (Perry et al., 2000). Ovariectomy in rats, which depletes endogenous estrogen levels, was also shown to induce hematopoietic defects and the presence of extramedullary hematopoiesis (Qiu et al., 2012) while murine ovariectomy was demonstrated to induce more short-term HSCs and led to increased engraftment in irradiated recipients (Li et al., 2013). These findings indicate that, while estrogen does appear to play a role in HSC homeostasis, its function remains controversial.

Xenoestrogenic Compounds are a Growing Problem in the Environment

In recent years, the number of xenoestrogenic compounds in the environment has greatly increased. While there are many different classes of xenoestrogens, some of the most common include mycoestrogens (fungal derived), phytoestrogens (plant derived), and synthetic estrogens (manufactured compounds) (Kerdivel et al., 2013). Both adult and embryonic zebrafish have long been known to be highly sensitive to the presence of estrogenic compounds as measured by induction of the estrogen target gene vitellogenin as well as through the use of estrogen reporter transgenic constructs as a readout of estrogen pathway activity. As a result, zebrafish have been utilized as sentinels to monitor water quality and environmental contamination, making them a particularly apt model system for studies on estrogen and xenoestrogenic regulation of development (Bakos et al., 2013; Carvan et al., 2000; Chen et al., 2010; Gorelick and Halpern, 2011).

While few studies have examined the effect of xenoestrogens directly on the hematopoietic system, at least one epidemiological study has indicated that exposure to xenoestrogenic flavonoid compounds during gestation is associated with an increased risk of Infantile Acute Leukemia (Ross, 2000) while other studies have suggested that exposure to estrogenic compounds such as genistein has the ability induce breaks in the Mixed Lineage Leukemia (MLL) gene similar to those seen during malignant transformation (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007). However, it remains to be determined if xenoestrogen exposure impacts hematopoietic homeostasis as well as if there are functional differences in the impact of short-term versus long-term exposure to these compounds on the blood system.

Summary of Thesis

Here, we identify multiple roles for estrogen in the regulation of the development and homeostasis of the blood system. When zebrafish are exposed to estrogen during early embryonic development, specification of hemogenic endothelium and the HSC niche is impaired via antagonism of somitic-derived VEGF. Conversely, inhibition of endogenous estrogen signaling expands the region of endothelium that is hemogenic and increases the number of functional HSCs. Exposure of zebrafish embryos to excess estrogen after specification of the niche enhances the expression of HSC markers and increases the number of cycling cells in the AGM. Estrogen treatment similarly increases the number of erythroblasts, however, it impairs their differentiation into mature and functional erythrocytes; this effect appears to be conserved in a mammalian model of estrogen excess.

In adults, male and female zebrafish show differential regulation of the hematopoietic system, with adult females possessing more cycling cells in the kidney marrow. After ablation by irradiation, exposure to exogenous estrogen enhances the regeneration of the hematopoietic system in both male and female fish. Significantly, the recovery of hematopoietic progenitors was faster in female fish compared to males, suggesting differential regulation of regeneration between the sexes that appears to be dependent on estrogen. Together, these data identify several specific roles for estrogen in HSC biology, indicating that it is a critical regulator of hematopoietic stem cell function.

Chapter 2:
Estrogen Defines the Dorsal-Ventral Limit of VEGF Regulation to Specify the Location of the Hemogenic Endothelial Niche

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Attributions

Estrogen was identified as a modifier of *runx1/cmyb* expression by WG and TEN. KJC performed all experiments and analysis for this section unless otherwise noted. VE helped with morpholino injections, MKG with EIA assay, and MC with generation of the VEGF reporter construct. MCD and CCC performed initial dose identification and timing of treatments; SN performed bead implant studies. GF helped with genotyping of mindbomb embryos. WK provided time-course RNA and JMH helped with FACS analysis. SL assisted with genotyping of the *kdrl* and *plcg* fish. DG and MH provided *ERE:GFP* reporter fish, and NL provided *kdrl* and *plcg* mutant embryos.

Introduction

Hematopoietic stem cells (HSCs) are characterized by the life-long ability to self-renew and give rise to each differentiated blood cell lineage. In mice, the first definitive HSCs arise at embryonic day 10.5 from hemogenic endothelium on the ventral wall of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region (Dzierzak and Speck, 2008). While the major signaling cascades that control the specification of arterial identity are well elucidated (Lawson et al., 2001; 2002), less is known about the factors regulating the dorsal/ventral limits of hemogenic endothelial identity. Recent studies have implicated several pathways in patterning of the AGM and subsequent HSC emergence, acting in part through the polarized expression of signaling elements on the dorsal side (Clements et al., 2011; Wilkinson et al., 2009). However, the mechanism by which these various signaling cascades integrate to specify the limits of hemogenic endothelium remains an open question, as does the identity of any ventral-derived signals (Jaffredo et al., 2013). Additionally, data from both zebrafish (Burns, 2005; Kim et al., 2013) and mice (Robert-Moreno et al., 2008) indicates that production of HSCs within arteries may be coincident rather than required for hemogenic endothelial specification. Following niche assignment, the transcription factor *Runx1* is recognized for its critical and highly conserved role in HSC development (North et al., 1999; Okuda et al., 1996; Wang et al., 1996), where it is required for HSCs to "bud" from hemogenic endothelium (Chen et al., 2009). In zebrafish, HSPCs first emerge in an analogous region of the dorsal aorta between 30-36 hours post fertilization (hpf) and *runx1* mediates a similar role in their production (Kissa and Herbomel, 2010). Our group has previously identified novel regulators of vertebrate HSC development via an *in vivo* chemical screening approach in zebrafish (Goessling et al., 2009; 2011; North et al., 2007;

2009); estrogens and estrogen-related compounds were also found in that screen to have a potent impact on the formation of *runx1+* HSCs.

Estrogen is a cholesterol-derived steroid hormone synthesized from testosterone by the enzyme CYP19A1 (Aromatase). There are three primary forms of estrogen found throughout the vertebrate phylum: estrone, estradiol, and estriol. 17β-Estradiol (E2), commonly referred to as "estrogen", is the most potent. Classically, E2 acts as a transcription factor upon binding to cytoplasmic nuclear hormone receptors, estrogen receptor 1 (Esr1; ERα) or Esr2 (ERβ), which subsequently translocate into the nucleus and bind estrogen response elements (EREs) in regulatory regions of estrogen-responsive genes (Heldring et al., 2007). In zebrafish, due to a genome duplication in recent evolutionary history, in addition to *esr1*, there are two *esr2* receptors: *esr2a* and esr*2b* (Menuet et al., 2002). E2 is also a ligand for a less well-characterized G-protein coupled receptor (GPER; also called GPR30) in mice and zebrafish (Liu et al., 2009; Revankar et al., 2005). While the role of E2 in reproductive organ development is established (Wilson and Davies, 2007), little is known about its potential impact on the formation or specification of other organ systems.

Endogenous E2 levels are highly variable during mammalian gestation. E2 levels are low during early pregnancy but increase throughout gestation, peaking just prior to delivery (Tulchinsky et al., 1972). It is unclear whether the developing embryo or fetus is exposed to increasing concentrations of E2; indeed, several pieces of evidence suggest mechanisms are in place to limit E2 exposure to the conceptus. Expression of 17β-hydroxysteroid

dehydrogenase type 2, which degrades E2, varies between umbilical arteries and veins and may protect the developing embryo from deleterious effects of excess maternal E2 (Simard et al., 2011). Surfeit estrogen can have a negative impact on maintenance of pregnancy, indicating a need for careful control over estrogen levels during gestation (Mahendroo et al., 1997). Based on the presumed importance of controlled exposure to E2 during embryogenesis, there are increasing concerns regarding the presence of estrogenic substances in the environment and the potential for long-term detrimental effects. Diethylstilbestrol (DES), a synthetic estrogen previously prescribed as an anti-abortifactant, was shown to cause increased risk of vaginal and cervical cancer as well as male genital defects in offspring whose mothers took the drug (Harris and Waring, 2012). Maternal hormonal use during the first trimester of pregnancy is associated with increased risk of infant acute leukemia, indicating that *in utero* exposure to estrogenic compounds may have significant effects on fetal hematopoietic homeostasis (Pombo-de-Oliveira et al., 2006). As little is known about the impact of estrogens on hematopoiesis during embryogenesis, we sought to prospectively determine the effect of estrogens and related compounds on the formation of HSCs.

Here, we demonstrate that exposure to excess E2 from early somitogenesis until 24hpf, the window of hemogenic endothelial specification, significantly decreased the formation of *runx1+* HSPCs in the AGM. In contrast, later exposure, during HSC specification and budding, enhanced HSPCs. The loss following early E2 exposure was mediated through Esr2 and resulted from a failure to specify hemogenic vascular endothelium in the dorsal aorta. Defects in both VEGF and Notch signaling, required for the establishment of arterial

identity and hemogenic niche formation, were noted following E2 treatment; hyperactivation of VEGF rescued *runx1* expression through induction of the notch pathway, indicating disruption of this signaling cascade underlies the observed hemato-vascular alterations. Exposure to xenoestrogens ethinylestradiol and genistein partially replicated E2-mediated phenotypes, decreasing *runx1+* HSPC expression and altering vascular niche specification. Significantly, antagonism of intrinsic estrogen signaling enhanced the expression and regulatory function of *VEGF*, increasing the zone of *scl+* hemogenic endothelium specification, independently of *ephrinB2+* arterial assignment, and enhancing the production of HSPCs. Together, these data suggest endogenous E2 acts as a previously unappreciated morphogen opposing the action of VEGF to control the assignment of the embryonic hemogenic endothelial niche.

Results

17β-Estradiol Decreases Formation of HSCs

E2 and estrogen-related compounds were identified through a prior chemical genetic screen for novel modulators of HSC formation (North et al., 2007) (**Figure 2.1 A**). To confirm and characterize the impact of hormonal regulation on hematopoiesis, zebrafish embryos were exposed to 17β-estradiol (E2) in the fish water from 5 somites (12hpf) until 36hpf, the window of hematopoietic initiation in zebrafish (**Figure 2.1 B)**. Markedly decreased combined expression of conserved HSPC markers *runx1* and *cmyb* was observed in the AGM following E2 treatment relative to untreated sibling controls (**Figure 2.1 C**) by whole mount *in situ* hybridization (WISH); penetrance of the effect was dose-dependent over a range of 0.1 to 10 μM E2 in the fish water, in the absence of toxicity, with the

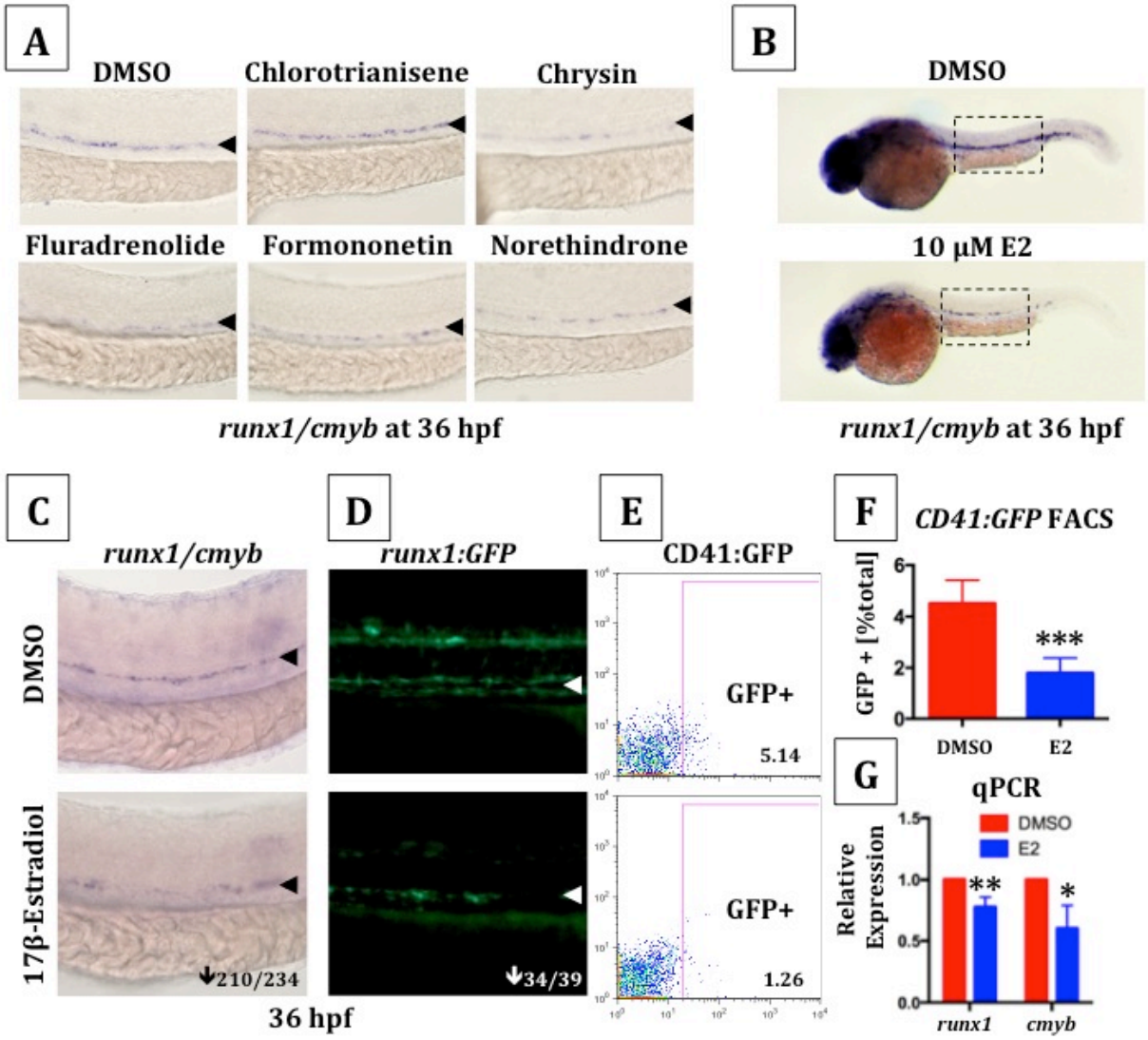


Figure 2.1

A. Reduced HSPC phenotypes following treatment with estrogenic compound "hits" from the chemical screen (10uM) as determined by *runx1/cmyb* WISH.
B. Whole mount embryos showing the effect of E2 on *runx1/cmyb* expression at 36hpf; the AGM region is indicated by a dashed-line box.
C. Exposure to E2 from 5 somites to 36hpf decreased WISH expression of *runx1/cmyb* in the AGM (210/234).
D. E2 diminished *runx1:GFP* fluorescence in the AGM of transgenic reporter fish (34/39).
E. FACS analysis of *CD41:GF*P (representative samples shown) indicated E2 reduced HSCs.
F. CD41:GFP was significantly decreased by E2 (n=20, 2-tailed t-test, p<0.001).
G. qPCR further quantified the reduction in *runx1* and *cmyb* by E2 (mean of triplicate experiments +/- SEM; 1-tailed t-test *runx* ** p<0.01, *cmyb* * p<0.05.)

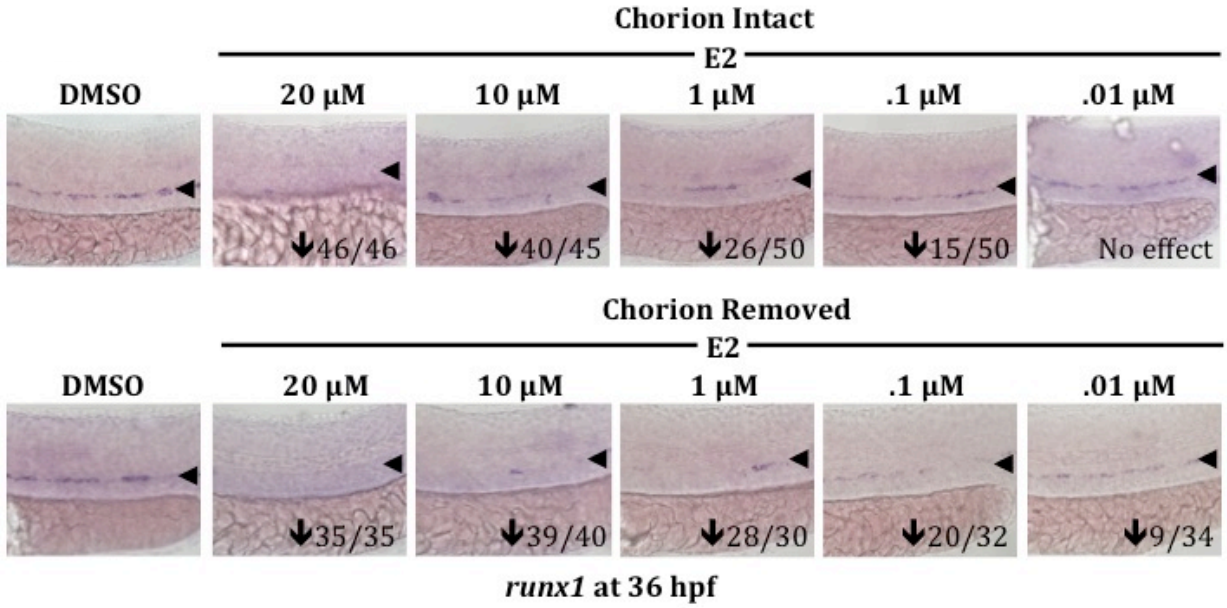


Figure 2.2

Effects on *runx1* WISH show dose-dependent penetrance over a range of 0.1 to 10 μM E2 in the fish water with the embryonic chorion intact (upper panels), and from 0.01 with it removed (lower panels); toxicity is observed at 20 μM E2.

embryonic chorion intact, and present with the chorion removed at 0.01 µM E2 (**Figure 2.2**). Using the transgenic *runx1*-reporter line (*Tg(runx1P1:eGFP)*), the reduction in Runx1+ cells by 10 µM E2 was confirmed *in vivo* (**Figure 2.1 D**); FACS quantification of the *CD41:GFP* line (*Tg(-6.0itga2b; (CD41):eGFP*) which marks mature HSCs indicated a similar decrease (**Figure 2.1 E-F)**; $p<0.001$)**.** These findings were corroborated by RT-qPCR on whole embryos (**Figure 2.1 G**; *runx* $p<0.01$, *cmyb* $p<0.05$). To determine if E2 regulation was receptor mediated, embryos were treated with a pan-Esr antagonist, fulvestrant (FULV; 15 µM), both in the presence and absence of E2 (**Figure 2.3 A**). Exposure to FULV increased *runx1*/*cmyb* alone, and restored expression in the majority of E2-treated embryos.

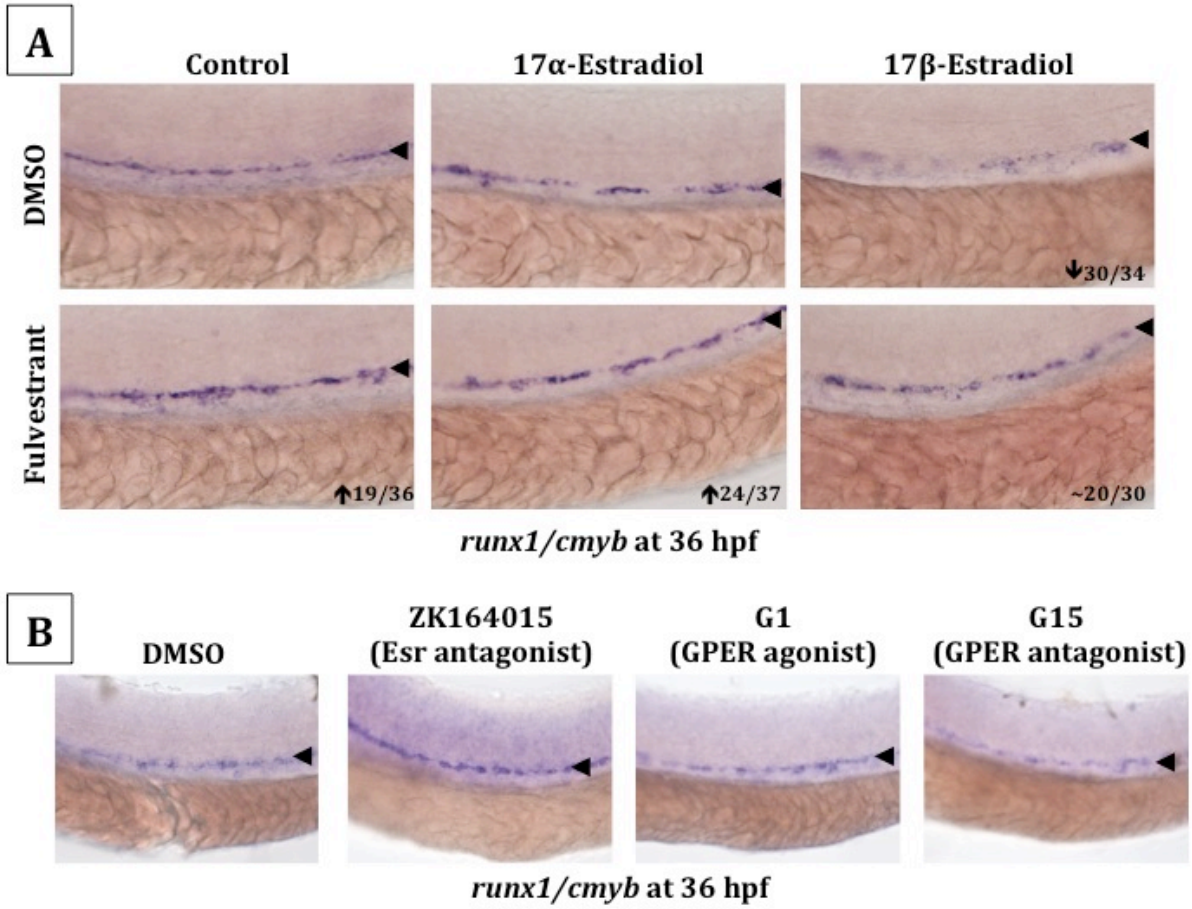


Figure 2.3

A. The pan-Esr antagonist fulvestrant blocked the effect of E2 exposure; the isomer 17α-Estradiol (10 uM) had no effect (n≥30/treatment).
B. Treatment with the pan-Esr antagonist ZK164015 (10 uM) enhanced *runx1/cmyb* expression while the GPER agonist G1 (1 uM) and GPER antagonist G15 (1 uM) had no impact on HSPCs (n≥15/treatment).

Identical results were observed with the pan-Esr antagonist, ZK164015 (ZK; 10 μM) **(Figure 2.3 B).** Notably, exposure to 17α-estradiol, the inactive isomer of E2, did not affect HSPCs (**Figure 2.3 A**), nor did treatment with the GPER agonist, G1, or antagonist, G15 (**Figure 2.3 B**). Finally, no alterations in gross development of other major embryonic organ systems were observed (**Figure 2.4**), together indicating that E2 acts via the cytoplasmic nuclear hormone receptors to impact HSPC formation during this window of embryogenesis.

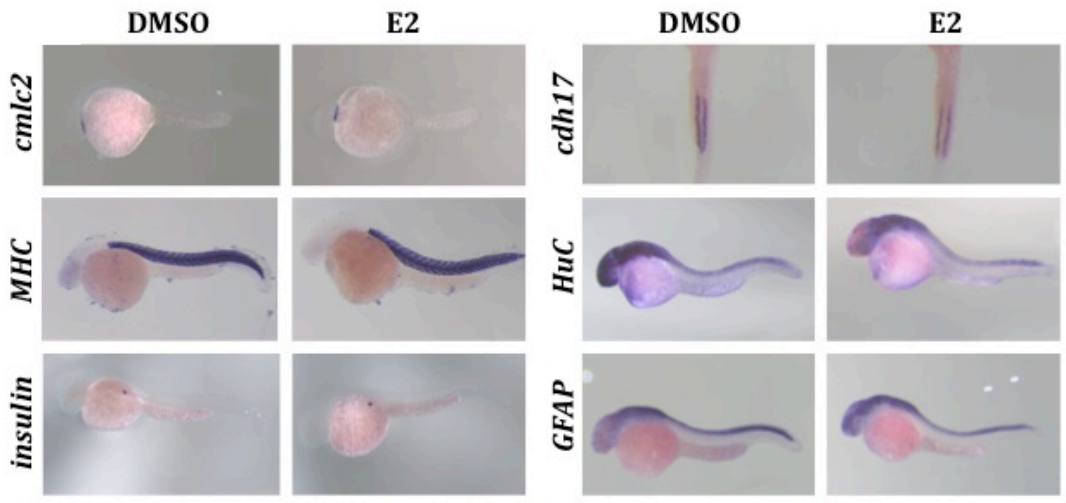


Figure 2.4

Other embryonic organ systems including heart (*cmlc2*), muscle (*MHC*), neural tissues (*HuC and GFAP*), kidney (*cdh17*) and endoderm (*insulin*) were not grossly impacted by E2 exposure.

Zebrafish possess endogenous estrogenic activity and the ability to respond to E2 signaling

To examine endogenous E2 content during hematopoiesis, a modified ELISA assay was performed. Zebrafish embryos show higher E2 levels early in development (0-48 hpf) than during the transition to larval stages (72-120 hpf) **(Figure 2.5 A).** The decrease in E2 during embryogenesis led to the hypothesis that endogenous estrogen may initially be contained in the yolk; following manual deyolking at 18hpf, E2 levels were found to be 4.7-fold higher in yolk compared to the body of the embryo ($p<0.05$). Consistent with maternal contribution of E2, enzymes involved in E2 synthesis were undetectable by qPCR until ~20hpf (**Figure 2.5 B**). As endogenous E2 was present, expression of the *esrs* was also assessed. Whole-embryo qPCR confirmed previously published results (Chandrasekar et al., 2010): *esr1* was expressed at very low levels immediately after fertilization but increased starting at 12hpf. In contrast, *esr2a* and *esr2b* were more robustly expressed immediately after fertilization and subsequently decreased, consistent with maternal deposition **(Figure 2.5 C);** after 12hpf, expression of *esr2a* and *esr2b* also increased, suggestive of embryonic transcriptional initiation. *esr2a* was previously shown to be expressed ubiquitously during the time frame of hematopoietic development while neither *esr1* nor *esr2b* were observed at detectable levels via WISH until at least 36 hours (Bertrand et al., 2007b). To identify whether hemato-vascular populations in the AGM region express *esrs*, we analyzed publicly available microarray data (Weber et al., 2005): both endothelial cells and emerging HSPCs express *esrs* to varying degrees, indicating they possess the capacity to respond to E2 regulation during embryogenesis (**Figure 2.5 D**).

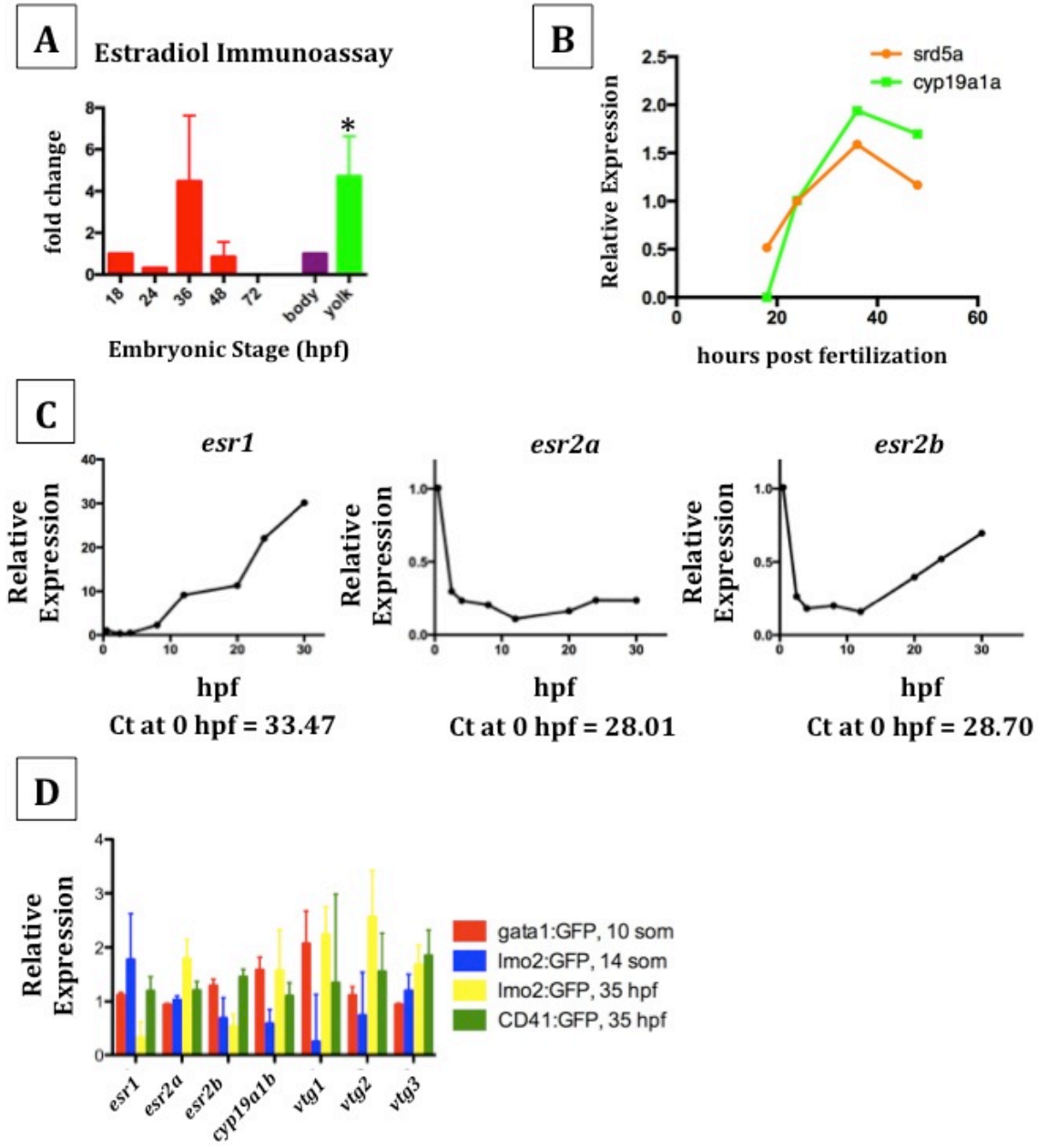


Figure 2.5

A. Estradiol immunoassays revealed endogenous estrogens are present during hematopoiesis; E2 was enriched 4.7 fold in the yolk versus the body of 18 hpf embryos (mean of triplicate experiments +/- SEM; 2-tailed t-test, * $p<0.05$).
B. qPCR analysis for *srd5a* and *cyp19a1a*, which regulate E2 production, indicated they are not expressed in early embryonic development but initiate at later stages.
C. qPCR analysis revealed expression of *esr1, 2a* and *2b* is dynamic over the course of hematopoietic development.
D. Reanalysis of published microarray data revealed *esrs* are expressed in hematopoietic and vascular lineages during primitive and definitive hematopoiesis.

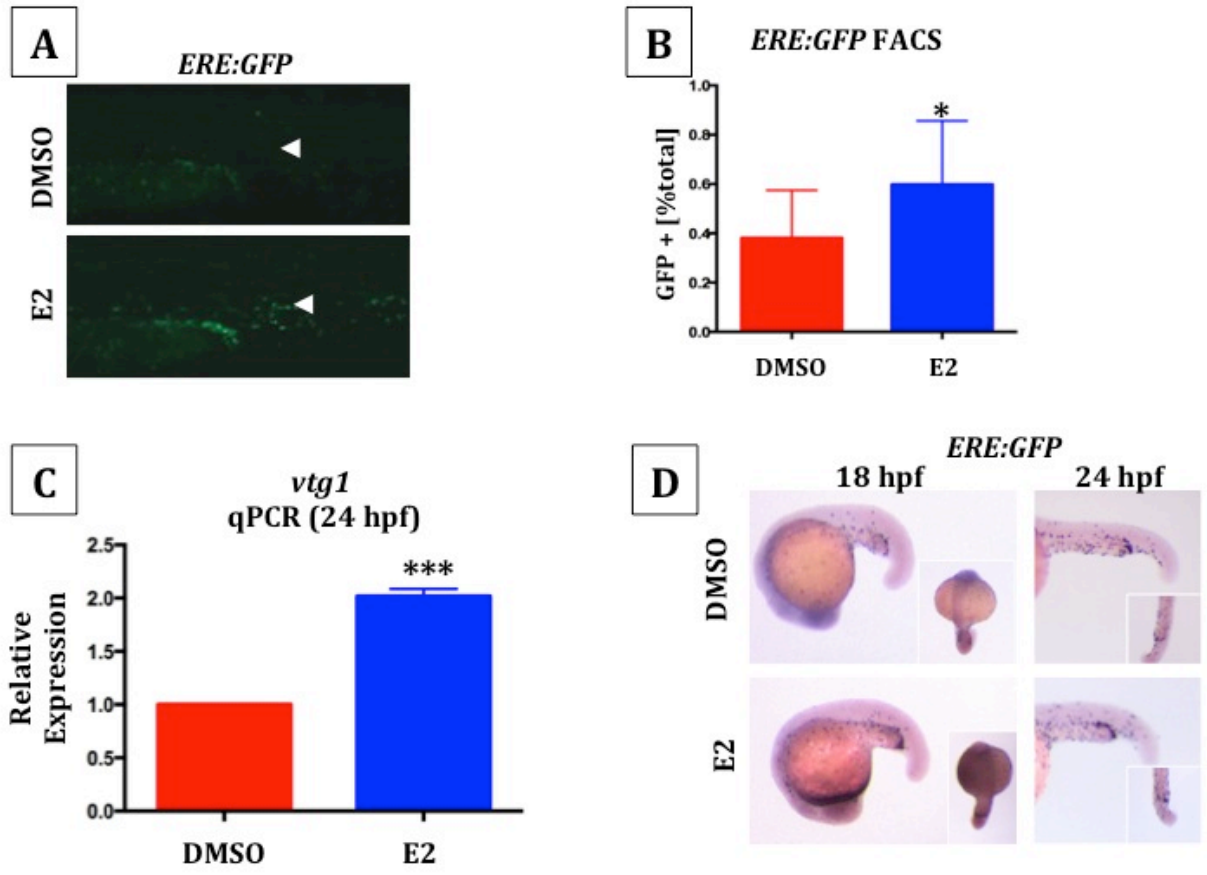


Figure 2.6

A. Estrogen Response Element:GFP fish (*ERE:GFP*) show estrogenic activity in the AGM region.
B. GFP expression (as seen in Figure 2.6 A) was increased 1.57-fold over baseline upon exposure to E2 (n=15, 2-tailed t-test, $p<0.05$).
C. qPCR analysis for *vtg1* reveals a 2-fold induction following E2 exposure (12-24hpf) compared to sibling controls (mean of triplicate experiments +/- SEM, one-tailed t-test ***$p<0.001$).
D. *gfp* WISH on *ERE:GFP* embryos revealed endogenous estrogen activity as early as 18 hpf, which could be enhanced by E2 treatment (n≥20/treatment).

To determine if E2-signaling was active in the AGM region, we examined estrogen activity in transgenic embryos expressing GPF under the control of an estrogen response element (ERE) *[Tg(5xERE:GFP)]* (Gorelick and Halpern, 2011). At 36 hpf, endogenous E2 activity was observed over the yolk sac extension, with the strongest signal situated to the ventral side of the embryo (**Figure 2.6 A**). Addition of 10 μM E2 increased whole-embryo *ERE:GFP* expression 1.57-fold, as quantified by FACS analysis, within the physiological range of estrogen activity (**Figure 2.6 B**; $p<0.05$) and consistent with recent reports (Hao et al., 2013); expression of vitellolenin-1 (*vtg1*), an endogenous estrogen target, showed a similar 2-fold induction ($p<0.001$) **(Figure 2.6 C)**. *ERE:GFP* WISH analysis revealed E2 activity over the yolk and in the ventral aspect of the developing trunk as early as 18 hpf **(Figure 2.6 D).** To ensure GFP expression was due to endogenous E2 and to identify the receptor(s) responsible for the hematopoietic phenotype, we utilized morpholino (MO)-mediated knockdown strategies. Injection of *esr* MOs (Froehlicher et al., 2009; Griffin et al., 2013; Pang and Thomas, 2010) into *ERE:GFP* embryos confirmed each reduced E2 activity similarly to ZK **(Figure 2.7 A-B)**. No effects on HSPCs were observed for *esr1* knockdown (**Figure 2.7 C**). While injection of *esr2a* MOs increased *runx1*/*cmyb* expression, E2 remained capable of reducing the appearance of HSPCs; *esr2b* knockdown alone had only mild effects on HSPCs, however, co-injection of MOs against *esr2a* and *esr2b* blocked the effect of E2, indicating *esr2(a+b)* is the primary mediator of the HSPC phenotype. These results were corroborated using receptor-selective compounds for Esr1 (PPT (agonist) and MPP (antagonist)) and Esr2 (DPN (agonist) and PHTPP (antagonist)) (**Figure 2.7 D** and *data not shown*). As microarray analysis indicated *esr2a* was present in endothelium (**Figure 2.5 D**), *ERE:GFP* activity was examined in *lmo2:dsRed* embryos (**Figure 2.8 A**).

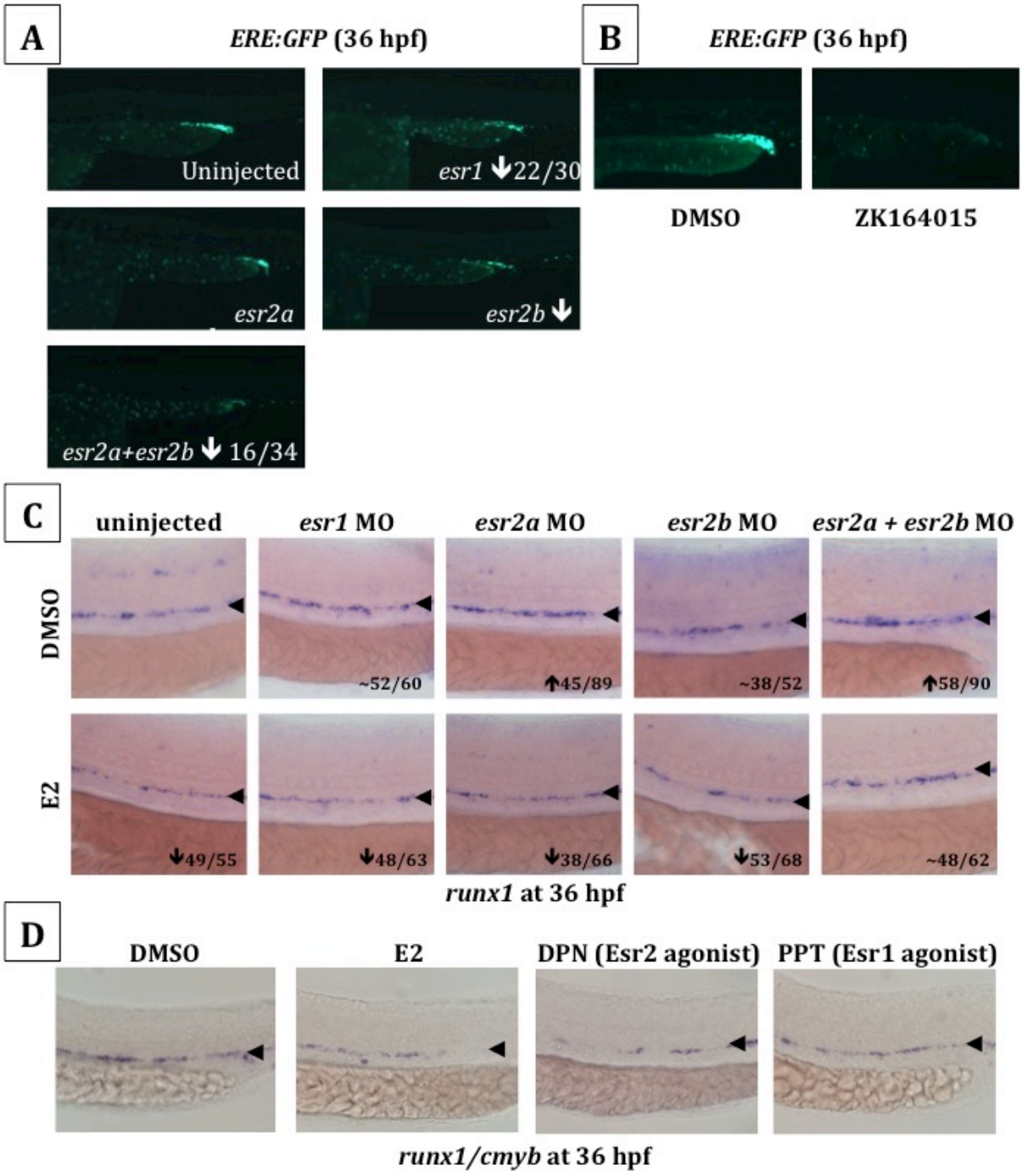


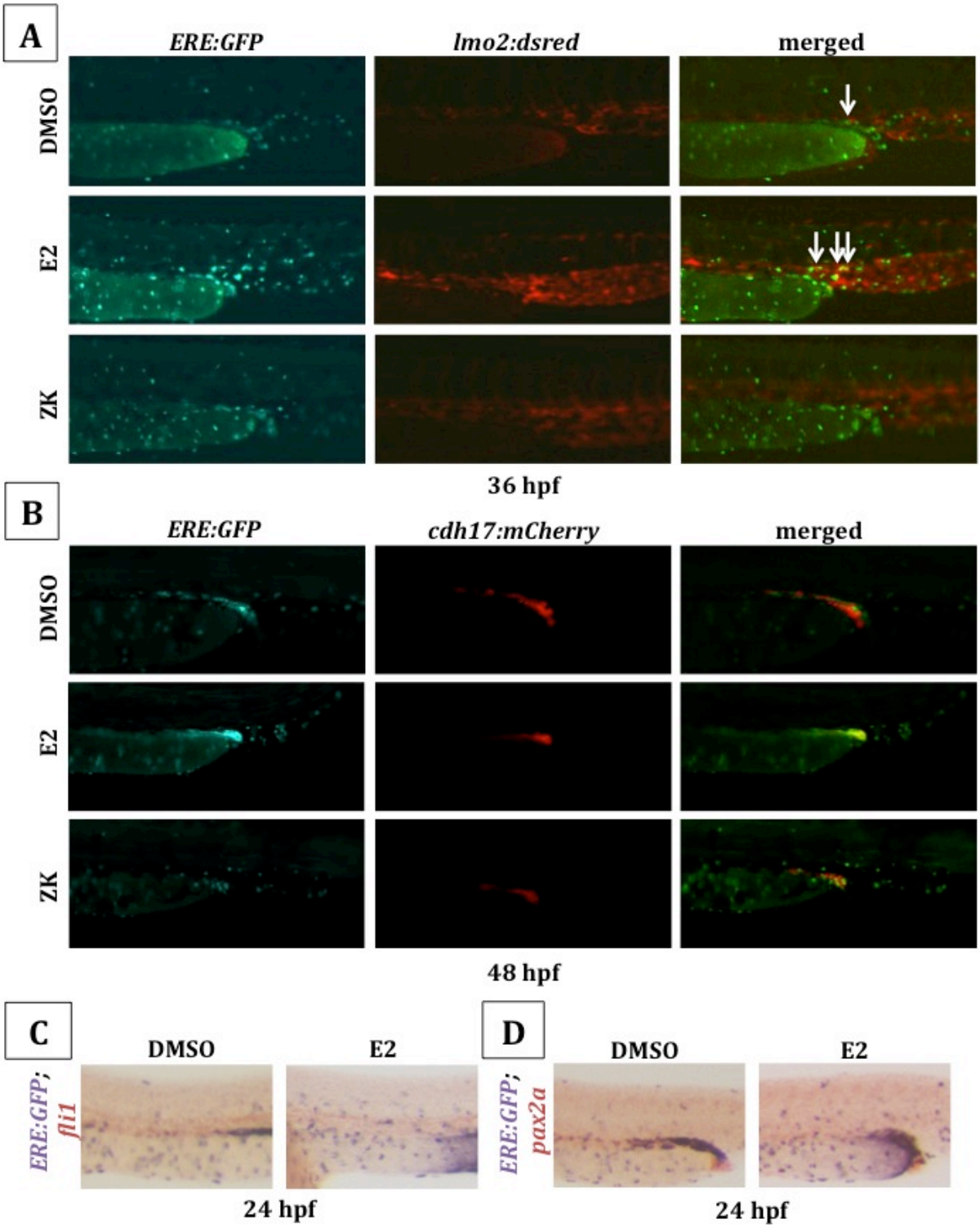
Figure 2.7

A. Morpholino knockdown of *esr1, 2a* and *2b* in the *ERE:GFP* background showed that each MO reduced estrogen activity, confirming specificity (n≥30).
B. Exposure of *ERE:GFP* embryos to ZK164015 decreased GFP expression (n≥20).
C. MO indicated the effect of E2 is mediated through *esr2a+b* as determined by *runx1/cmyb* WISH; *esr1*-MO had no impact on *runx1/cmyb* expression (n≥30).
D. Treatment with DPN (10uM), an Esr2 agonist, mimicked E2 and decreased expression of *runx1/cmyb*, while PPT (20 uM), an Esr1 agonist, had no effect.

Figure 2.8

A. *ERE:GFP* embryos show low levels of GFP in the developing trunk vasculature, labeled by *lmo2:dsRed*; the region of GFP expands following E2 exposure and is reduced by treatment with the Esr antagonist ZK164015 (n≥20).
B. *ERE:GFP* embryos exhibit estrogen activity in the developing mesonephros as indicated by *cdh17:mCherry* at 48hpf, which responds to alterations in estrogen signaling (n≥20).
C. Occasional vascular co-expression of *fli1* (red) and *gfp* (purple) in *ERE:GFP* zebrafish was noted at 24 hpf, particularly after E2 exposure.
D. *gfp* (purple) expression in *ERE:GFP* zebrafish co-localized with the kidney marker *pax2a* (red) as early as 24 hpf, both in the presence and absence of E2 treatment.

Figure 2.8 (Continued)



Occasional *ERE:GFP*+ cells were embedded in the aortic vasculature of untreated controls at 36 hpf, and the number of double positive cells increased following E2 exposure; WISH indicated vascular (*fli1+*) localization and response to E2-stimulation could be detected as early as 24 hpf **(Figure 2.8 C).** Strong E2-responsive *ERE:GFP* co-localization was also noted in the kidney, ventral to the aortic vasculature and adjacent to maternal yolk deposits, using the Tg(*cdh17:mCherry*) reporter (**Figure 2.8 B**); kidney co-localization and response to E2 was observed as early as 24 hpf by WISH (**Figure 2.8 D**). Together, these data indicate E2 is present and can elicit receptor-mediated activity in the AGM during hemato-vascular development.

E2 Exposure Impairs Formation of the Hemogenic Arterial Hematopoietic Niche

In order to determine how the effect on HSPCs was mediated, we examined the impact of E2 exposure during two major developmental milestones of HSC production: from ~12-24 hpf, the hemogenic vascular niche is established, and from ~24-36 hpf, post-heartbeat initiation and circulation onset, HSCs are specified and begin to bud from hemogenic endothelium in the artery. When embryos were exposed to E2 during these discrete time windows, a biphasic effect was noted: exposure from ~12-23 hpf resulted in a marked absence of *runx1/cmyb* expression while treatment from ~26-36 hpf increased HSPC markers (**Figure 2.9 A**); DPN mimicked both aspects of this phenotype, suggesting each was mediated by Esr2 in the AGM (**Figure 2.9 B)**.

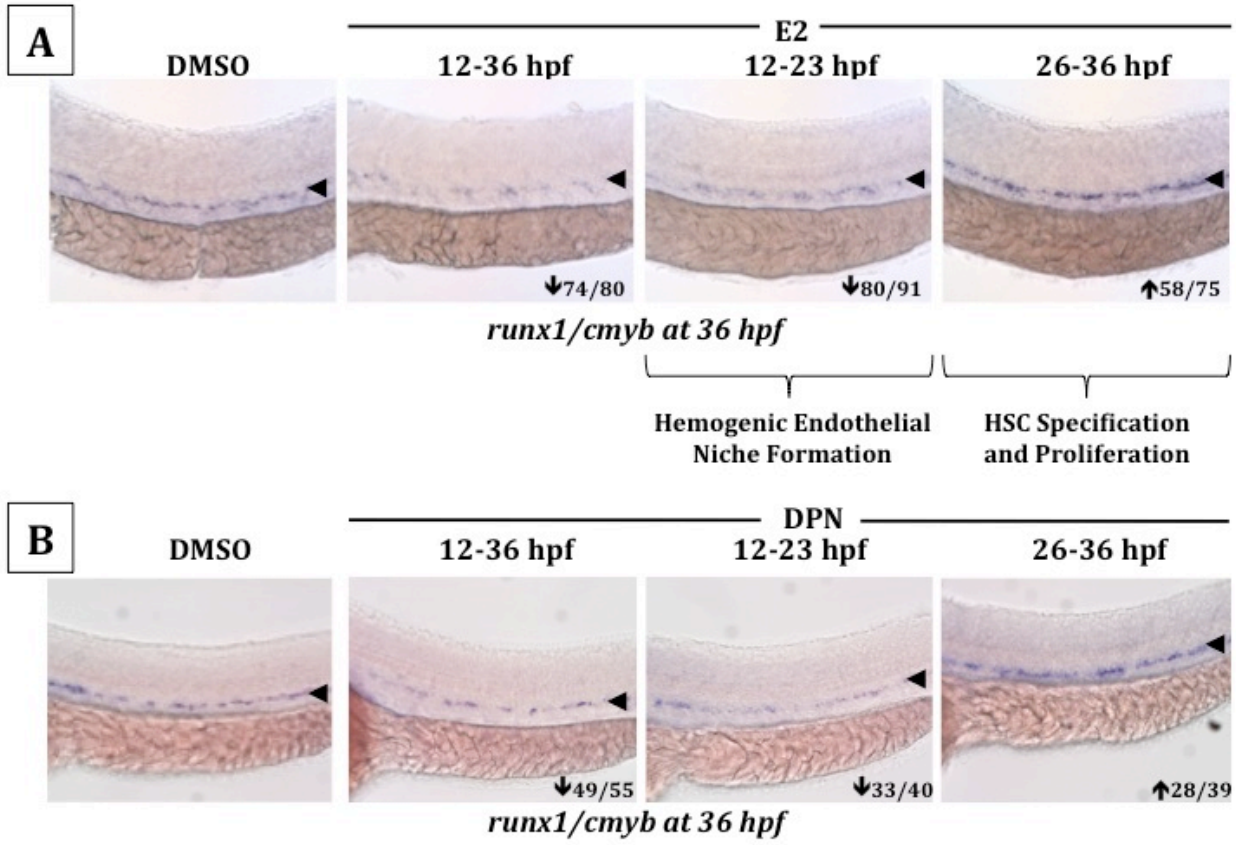


Figure 2.9

A. E2 had a biphasic effect on HSPCs: treatment during hemogenic niche specification (12-23 hpf) decreased *runx1* WISH while exposure during HSC specification and budding (26-36 hpf) increased HSPCs (n≥75/treatment).
B. The Esr agonist DPN had a biphasic effect on HSPC formation, reminiscent of E2, where treatment from 12-23 hpf decreased *runx1/cmyb* expression, while exposure from 26-36 hpf increased HSPCs (n≥39/treatment).

As the negative impact of E2 from 12-24hpf was the dominant phenotype, we sought to further characterize the impact of E2 exposure on the development of the hemogenic vascular niche. Analysis of embryos expressing GFP under the control of the angiopoietin receptor promoter, *tie2* (Tg*(Tie2:EGFP)* indicated normal circulation and proper formation of the dorsal aorta and cardinal vein at the doses of E2 utilized in these studies (**Figure**

2.10 A); in contrast, *tie2:GFP*+ intersomitic vessels (ISVs) were strongly reduced. Expression of the VEGF receptor *flk1* (*kdrl*, *VEGFR2*) was decreased in the major vessels of the majority of E2-exposed embryos and ISV expression reduced or absent at 36 hpf, which was confirmed by qPCR (**Figure 2.10 B,G;** $p<0.01$); however, the number of flk1+ cells, as assessed by FACS using the *flk1:GFP* line (Tg(*kdrl:EGFP*)) was not significantly altered (**Figure 2.10 F**) confirming gross vascular structure was unaffected by E2 treatment. To determine if the reduction in *flk1+* ISVs was indicative of a defect in vessel maturation, markers of artery-vein specification were examined: *ephrinB2* was robustly decreased by WISH and qPCR, while *flt4*, a venous marker, increased (**Figure 2.10 C-D, G;** *ephrinB2* $p<0.001$; *flt4* $p<0.05$) Finally, to assess if the subset of hemogenic endothelium was negatively impacted, *scl*-expressing cells in the transgenic Tg(-*6tal1:EGFP*) reporter were examined: following E2-exposure, arterial expression of *scl* was almost absent, as confirmed by qPCR ($p<0.05$) (**Figure 2.10 E,G**). To determine if *scl* loss was responsible for the dramatic reduction in HSPCs, E2-treated embryos were injected with *scl* mRNA **(Figure 2.10 H);** whereas 82% of E2-exposed embryos showed *runx1* loss, *scl*-injection rescued expression in 77% of treated embryos. Together, this data indicates E2 alters the production HSPCs via regulation of aortic vessel maturation and tissue specification. As early E2 exposure was observed to have a potent negative effect on the hemogenic endothelium, we sought to determine if the later effects of E2 on HSPCs were likewise due to niche regulation. The positive effect of E2 (24-36hpf) was confirmed by qPCR for *runx1* and *cmyb* (**Figure 2.11 A**; $p<0.05$), however, no impact was found on *ephrinB2* or *flt4* (*data not shown*), suggesting at later stages E2 may act directly on HSPCs. As E2 is a known cell

Figure 2.10

A. Treatment with E2 had no impact on axial vessel formation as assessed by *tie2:GFP* (n≥20).
B. E2 disrupts ISV formation as indicated by *flk1/kdrl* (*VEGFR2*) WISH (59/70).
C. Expression of arterial *ephrinb2* is robustly decreased by E2 (77/80); red arrowheads indicate artery, blue arrowheads vein.
D. WISH for venous *flt4* was increased after E2 exposure (40/53).
E. Expression of *scl:GFP* was reduced by E2 treatment (25/30).
F. FACS of *flk:GFP+* embryos (n=32) revealed no change in numbers of *flk+* cells by E2.
G. qPCR confirmed decreases in expression of *flk1*, *ephrinB2,* and *scl* expression following E2 treatment (mean of triplicate experiments +/- SEM; one-tailed t-test; *flt4* *p<0.05, *flk1* **p<0.01, *ephrinB2* ***p<0.001).
H. Injection of *scl* mRNA increased *runx1* expression in the AGM and rescued loss of *runx1* following E2 treatment as determined by WISH (n>55 per treatment).

Figure 2.10 (Continued)

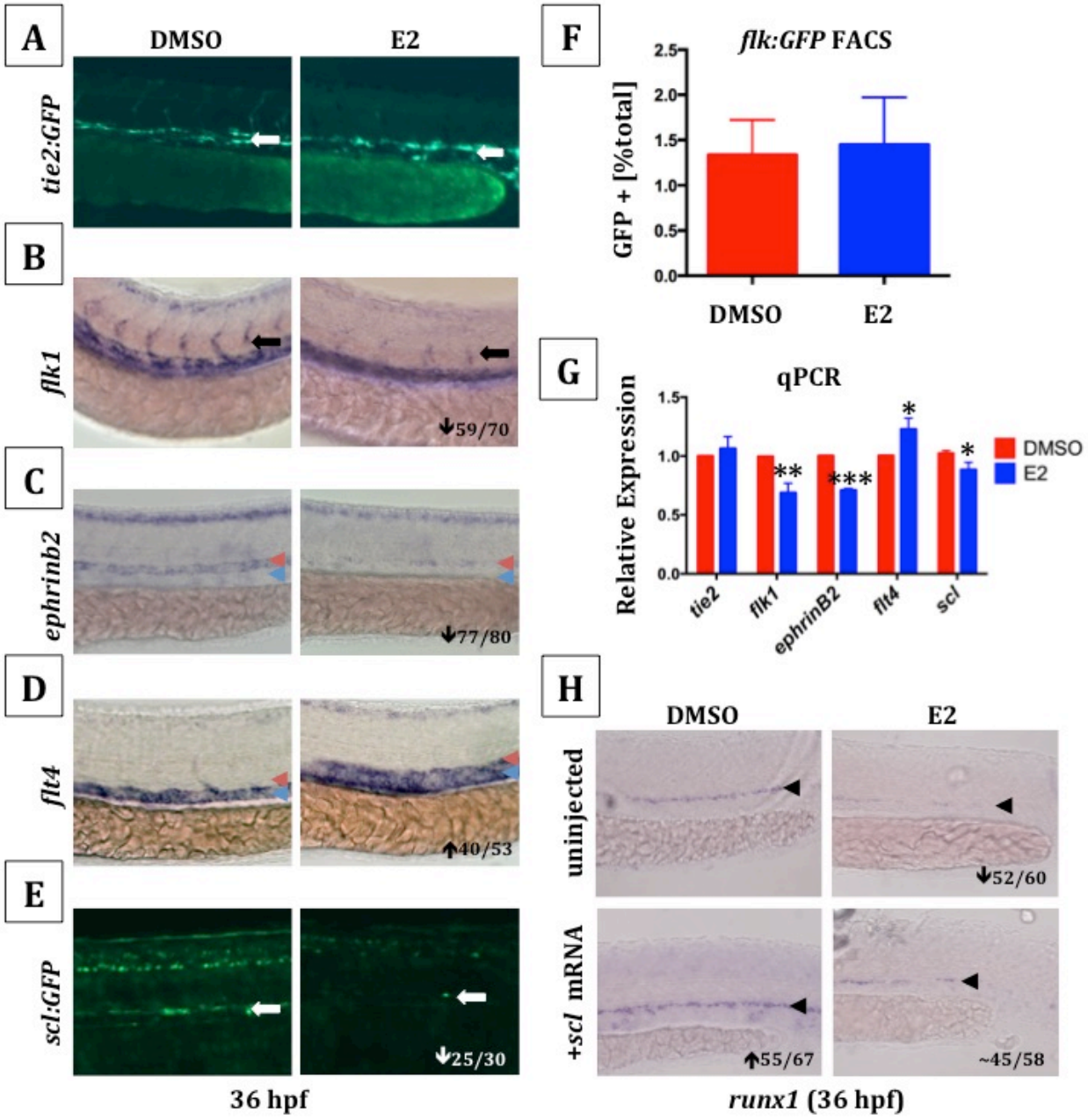
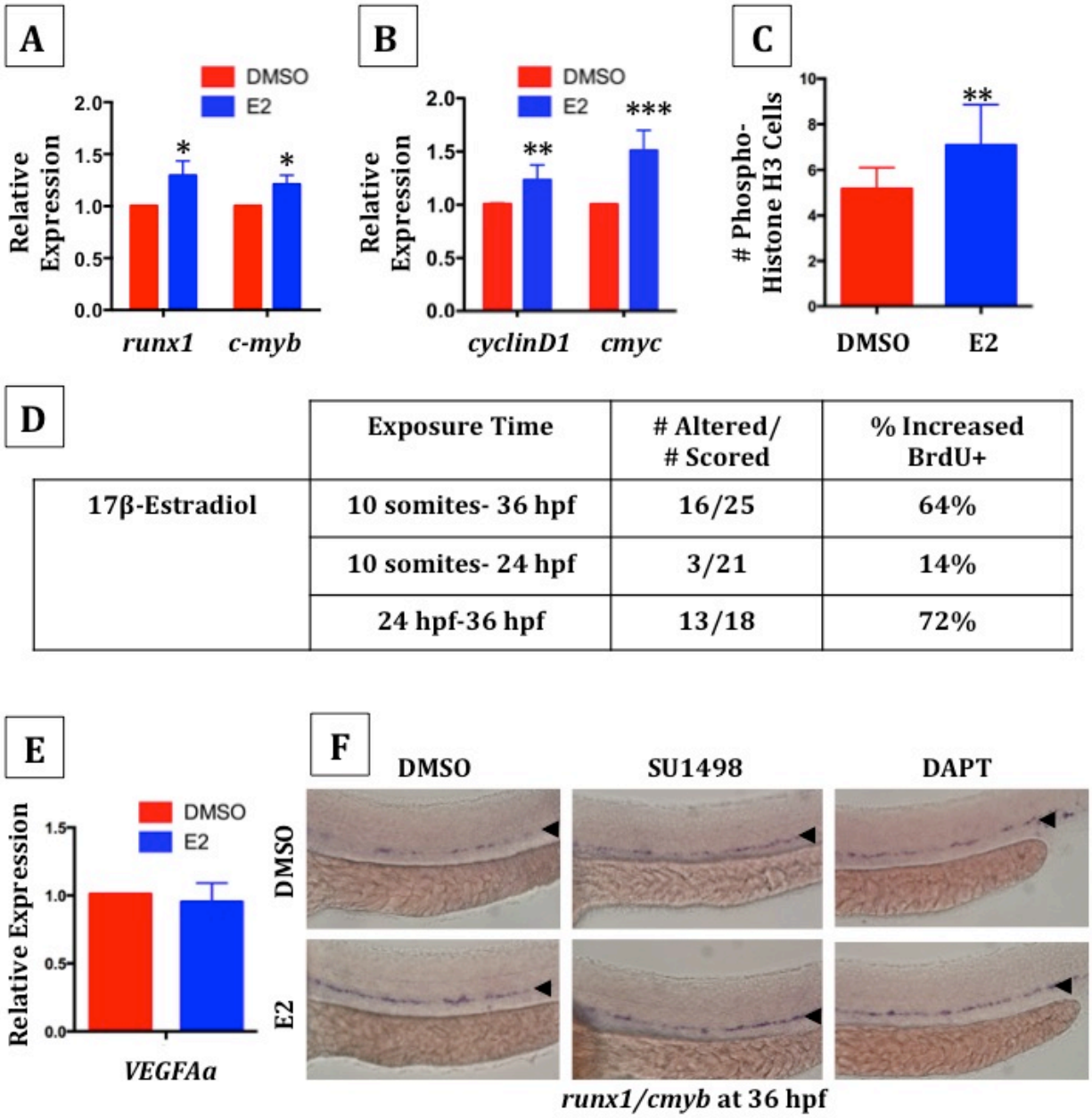


Figure 2.11

A. qPCR confirmed increased expression of *runx1* and *cmyb* after late (26-36hpf) E2 exposure (mean of triplicate experiments +/- SEM, one-tailed t-test *p<0.05).
B. *cyclinD1* and *c-myc* were elevated as determined by qPCR following late E2 treatment from 26-36hpf (mean of triplicate experiments +/- SEM; one-tailed t-test *cyclinD1* **p<0.01, *c-myc* ***p<0.001)
C. Phospho-histone H3 staining revealed increased cell cycling after E2 exposure (n=12/treatment; **p<0.01).
D. DNA synthesis as indicated by BrdU incorporation was increased in embryos exposed to E2 from 24-36hpf (n≥18).
E. qPCR revealed no change in VEGF expression following late E2 treatment (26-36hpf (mean of triplicate experiments +/- SEM).
F. Inhibition of VEGF or Notch signaling (SU1498 (10uM) and DAPT (20 uM), respectively) had no impact on HSPCs, nor was it able to block the increase in HSPCs mediated by E2 treatment from 26-36hpf.

Figure 2.11 (Continued)



	Exposure Time	# Altered/ # Scored	% Increased BrdU+
17β-Estradiol	10 somites- 36 hpf	16/25	64%
	10 somites- 24 hpf	3/21	14%
	24 hpf-36 hpf	13/18	72%

cycle regulator, several indicators of cell proliferation were examined: both *cyclinD1* and *c-myc* were increased by E2 exposure from 26-36hpf (**Figure 2.11 B** *cyclinD1* $p<0.01$, *c-myc* $p<0.001$); phosphohistone H3 (**Figure 2.11 C,** $p<0.01$) and BrdU incorporation (**Figure 2.11 D**) were similarly enhanced, together indicating elevations in *runx1+* HSPC*s* following later E2 treatment coincides with increased cell proliferation, consistent with recent reports (Nakada et al., 2014). In contrast, BrdU was not altered by early E2 exposure. In sum these data indicate that E2 only affects the vascular niche for a limited time (12-24 hpf), independent of proliferative activity, implying it may play a role in specifying hemogenic endothelium.

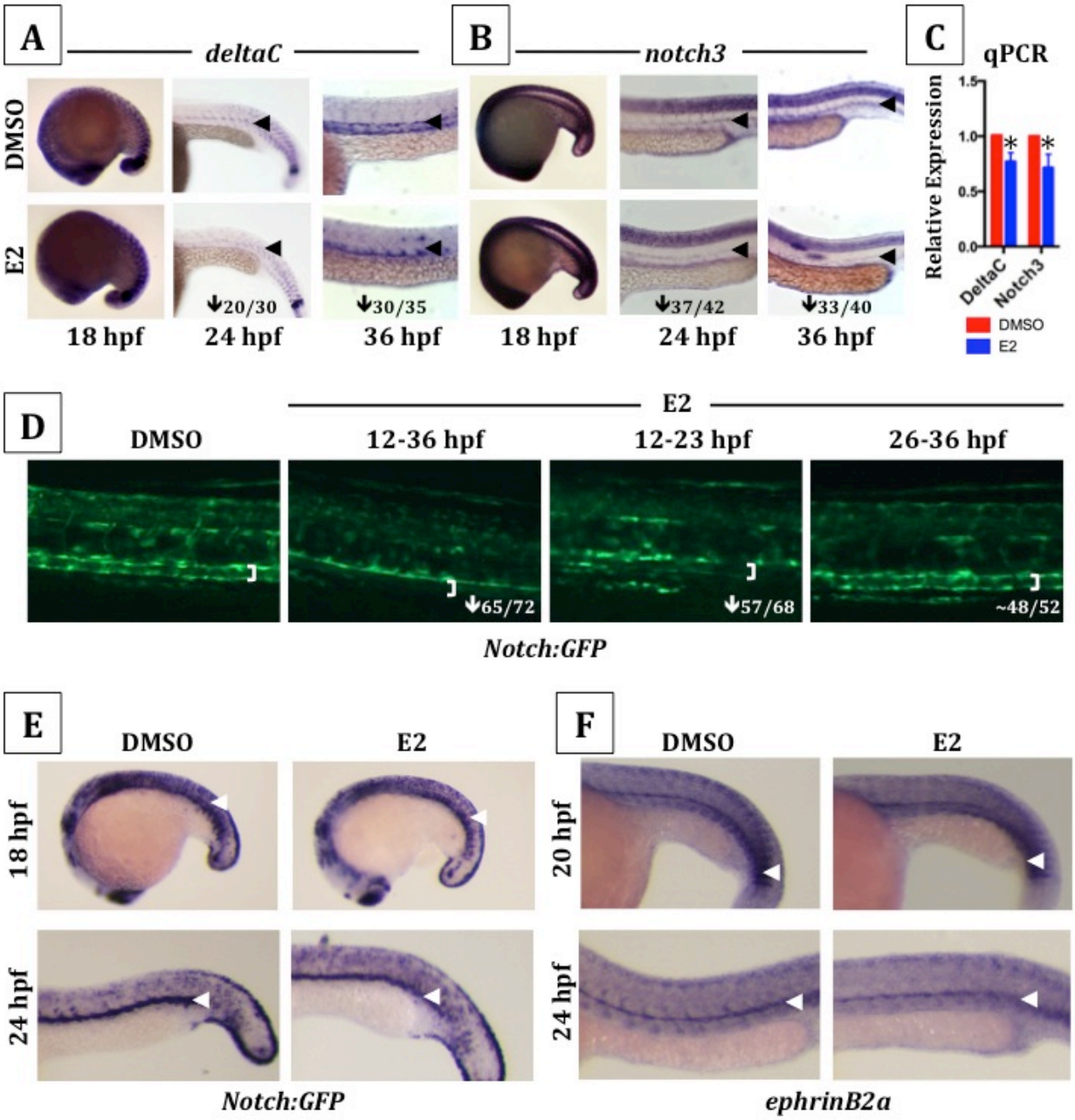
E2 Exposure Disrupts the VEGF/Notch Pathway Leading to Vascular Defects

Specification of the arterial trunk vasculature where the hemogenic endothelium is located occurs through a precise stepwise induction of signaling pathways, starting with Hedgehog (HH), followed by Vascular Endothelial Growth Factor (VEGF) and Notch (Lawson et al., 2001; 2002). As Notch is most proximal to HSCs, it was examined for alterations in response to E2. At 18 hpf, after 6hrs of E2 treatment, no changes in global expression of the Notch ligand *deltaC* were noted, indicating Notch signaling likely initiates correctly (**Figure 2.12 A**); this finding was corroborated by analysis of *Notch:GFP* (*Tg(EPVV:Tp1-MmuHbb:EGFP)*) reporter embryos which expresses GFP under control of the *RBP:jK* binding site (Parsons et al., 2009) and the notch target *ephrinB2a* (**Figure 2.12 E-F**). At 24 and 36 hpf, however, marked decreases in the arterial expression of *deltaC* were observed, which were confirmed by qPCR (**Figure 2.12 A, C;** $p<0.05$); a similar pattern of regulation

Figure 2.12

A. E2 exposure had no impact expression of *deltaC* by WISH at 18 hpf, but strongly reduced arterial expression at 24 and 36 hpf (n>30/treatment); arrowhead indicates artery.
B. Expression of *notch3* was similarly regulated by E2 treatment at 18, 24 and 36 hpf (n>30/treatment); arrowhead indicates artery.
C. qPCR confirmed E2-mediated reductions in *deltaC* and *notch3* expression at 36 hpf (mean of triplicate experiments +/- SEM; one-tailed t-test *deltaC, notch3* * $p<0.05$).
D. Decreased *Notch:GFP* was observed in the artery (white bracket indicates artery walls) after E2 treatment from 12-36 or 12-23 hpf; no change after E2 from 26-36 hpf (n≥52).
E. Notch activity was not altered at 18 hpf, but decreased as 24 hpf following E2 treatment (n≥25).
F. *ephrinB2* expression, normal at 20 hpf, was slightly decreased by E2 at 24 hpf (n≥20).

Figure 2.12 (Continued)



was observed for *notch3* (**Figure 2.12 B-C;** $p<0.05$). The *Notch:GFP* reporter confirmed strong reductions in arterial and intersomitic activity after E2 exposure (**Figure 2.12 D**); inhibition was also noted for the notch target *hey2* and the VEGF-regulated arterial marker and Notch pathway component *notch1b* (**Figure 2.13 A-B**). Given the Notch phenotypes, hyperactivation of the Notch pathway, downstream of ligand binding, was used to determine if restoration of Notch signaling could rescue E2-mediated HSPC defects. Heat-shock induction of *hs:Gal4;UAS:NICD* in controls resulted in increased *runx1* expression in both the artery and the vein (23/37), matching published results (Burns, 2005), while NICD activation in E2 treated fish recovered *runx1+* HSPCs (20/31) (**Figure 2.13 C**). *mindbomb* (*mib*) mutants have defective Notch signaling (Itoh et al., 2003); *mib+/-* embryos showed a previously unreported reduction in *runx1* expression compared to controls (**Figure 2.13 D**), which was exacerbated after E2 treatment such that HSPCs were virtually absent (22/41), mimicking the homozygous phenotype. These data indicate synergy between E2 exposure and loss of Notch activity, and further demonstrate deficits in Notch signaling contribute to the hematopoietic abnormalities induced by exogenous E2.

VEGF activity is considered to lie upstream of Notch in vascular specification. Following E2 exposure, somitic expression of *VEGFAa* was decreased at both 18 and 24 hpf, which was confirmed by qPCR (**Figure 2.14 A-B;** $p<0.001$). Heat-shock inducible VEGFAa embryos (*hs:VEGFAa* (Wiley et al., 2011)) were utilized to determine if hyperactivation of VEGF could rescue the E2-mediated HSPC defects. *VEGFAa* induction increased *runx1* expression (31/37) in controls (**Figure 2.14 C**), consistent with our recent observations (Harris et al., 2013) and compensated for the deleterious effect of E2 (16/33). However, when Notch

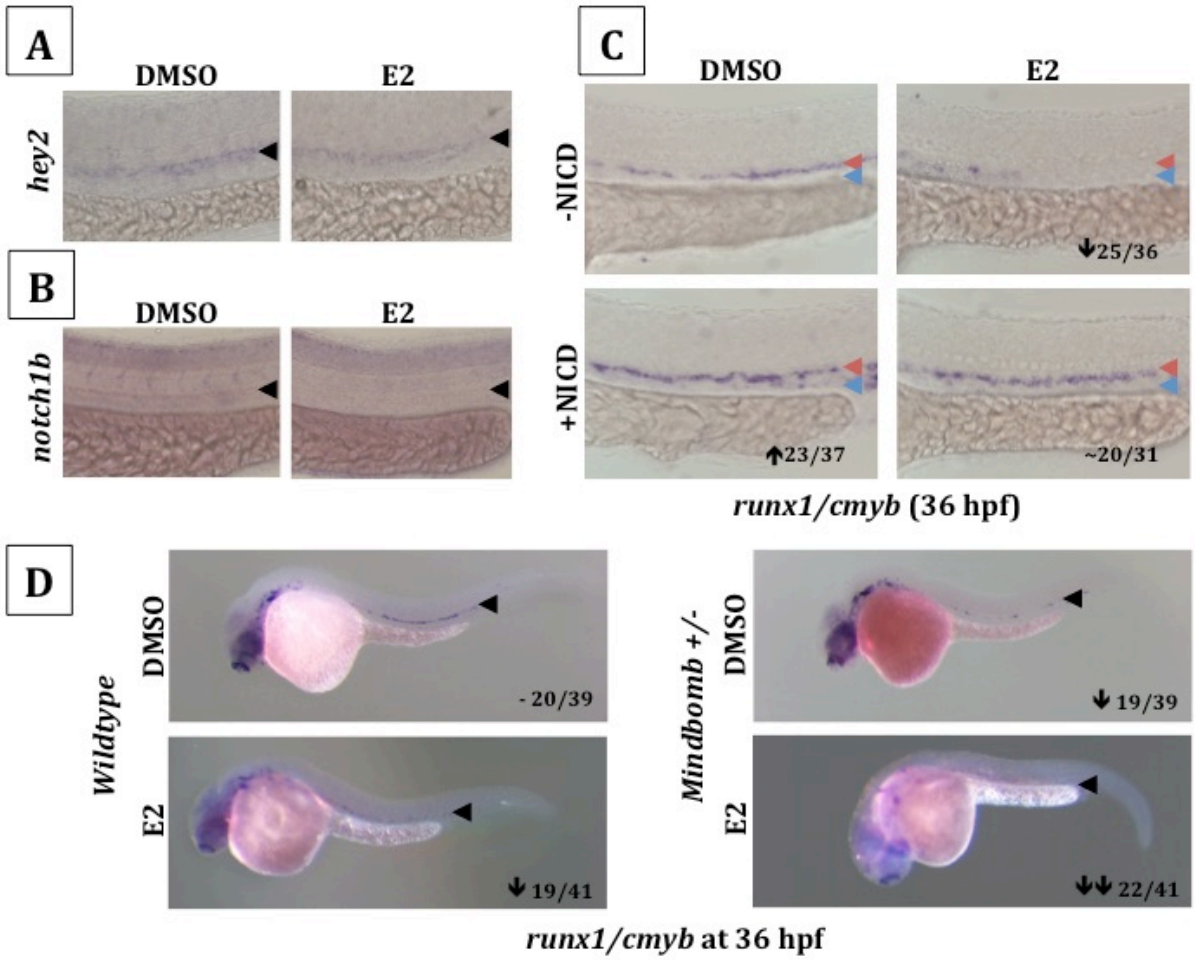


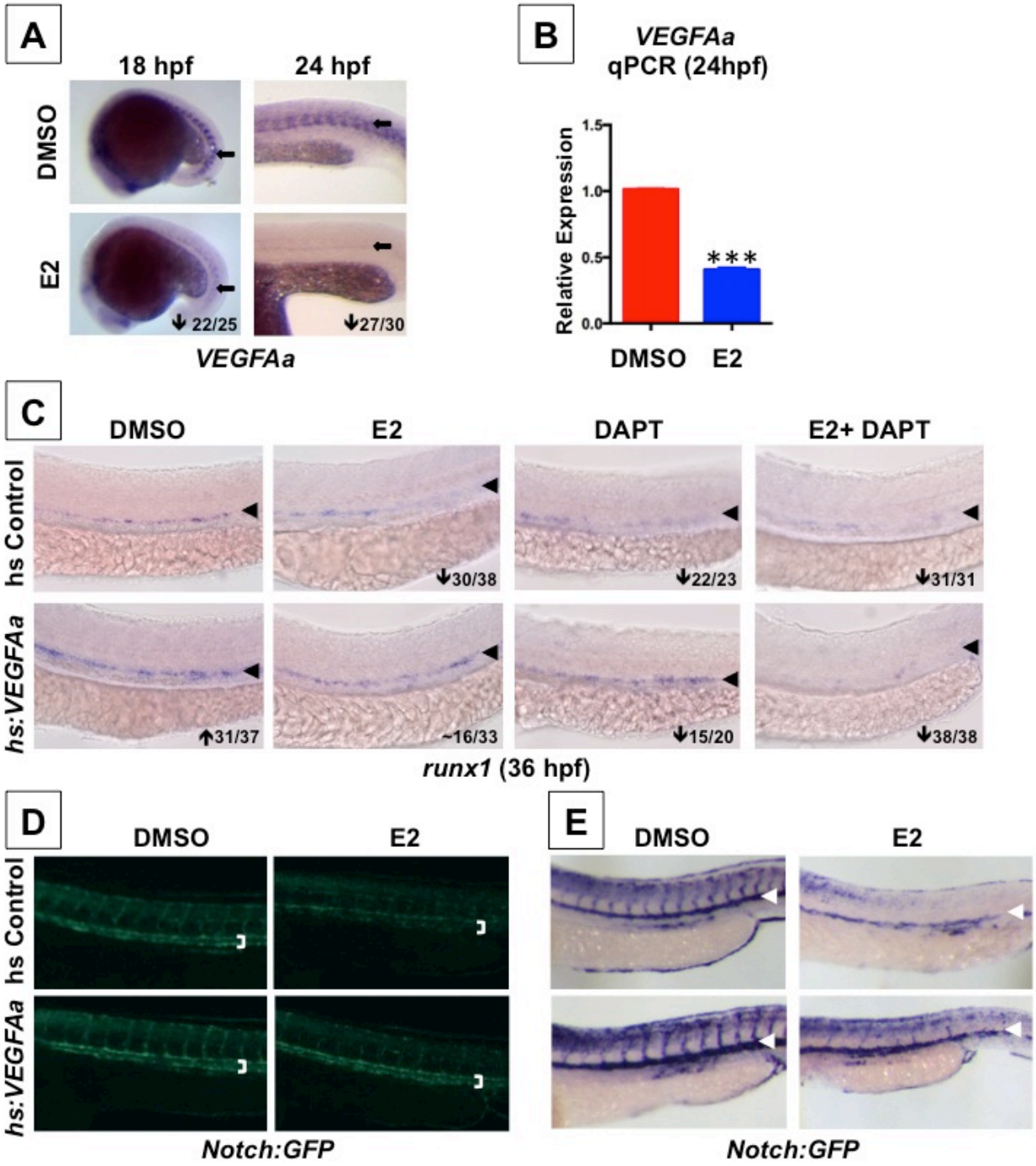
Figure 2.13

A. Expression of the Notch target *hey2* was reduced after E2 treatment (n≥20).
B. Arterial *notch1b* expression was drastically decreased by E2 exposure (n>20).
C. Hyperactivation of the Notch pathway by NICD induction increased expression of *runx1/cmyb* and rescued HSPCs in E2-treated embryos (n≥31/treatment).
D. *Mindbomb (mib+/-)* heterozygotes exhibit decreased expression of *runx1/cmyb* compared to sibling controls; E2 treated *mib+/-* embryos have an absence of HSCPs (n≥39).

Figure 2.14

A. Expression of *VEGFAa* was strongly decreased in zebrafish following E2 treatment at both 18 and 24 hpf (n≥25/treatment); arrow points to somite staining.
B. qPCR confirmed expression of *VEGFAa* was decreased at 24 hpf (mean of triplicate experiments +/- SEM; one-tailed t-test *** $p<0.001$).
C. Heat shock induction of *VEGFAa* increased *runx1* expression and rescued the effect of E2 exposure. Treatment with DAPT decreased expression of *runx1* and blocked the VEGF-mediated rescue of HSPCs (n≥20/treatment); arrowhead indicates artery.
D. *Notch:GFP* reporter activity was restored in E2 treated embryos by heat shock induction of *VEGFAa.*
E. *gfp* WISH confirmed restoration of Notch activity in the vasculature after *VEGFAa* induction in E2 exposed embryos.

Figure 2.14 (Continued)

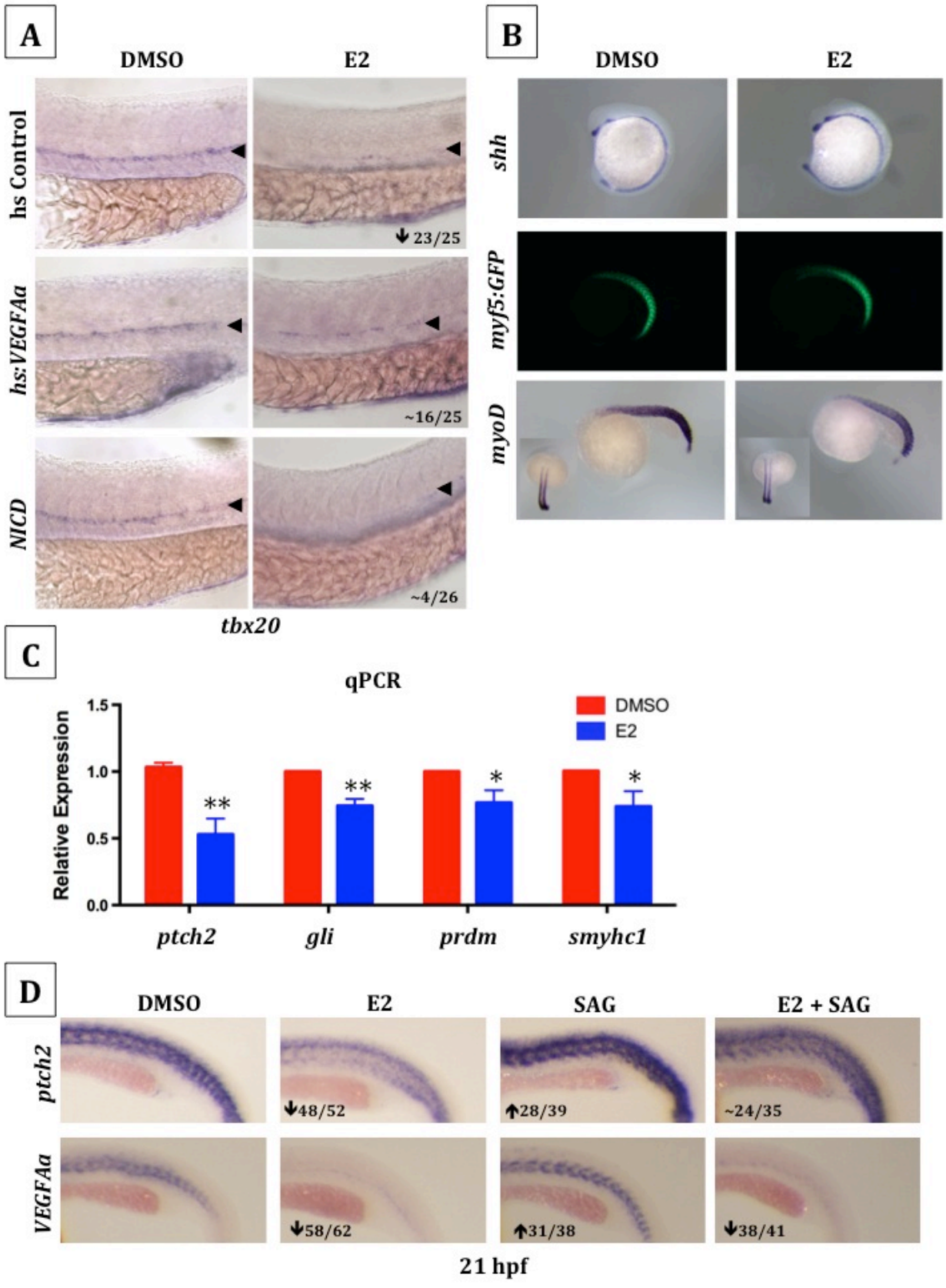


activity was simultaneously blocked by the gamma secretase inhibitor DAPT (20 μM), VEGF overexpression failed to rescue the effect of E2 on HSPCs (38/38) (**Figure 2.14 C**); this Notch dependence was confirmed by *Notch:GFP* WISH where an increase in activity was seen following *VEGF* induction (**Figure 2.14 D-E**). Expression of *tbx2*0 is unaffected in *mib* mutants, indicating it is regulated upstream of Notch signaling in arterial specification (Lawson et al., 2001). Arterial *tbx20* was reduced by E2 (23/25); induction of *VEGFAa* in E2 treated embryos partially rescued tbx20 expression (16/25) while *NICD* could not (**Figure 2.15 A**) (4/26). In contrast, effects of later E2 exposure (24-36) were independent of either Notch or VEGF **(Figure 2.11 E-F).** To assess if HH-signaling, upstream of VEGF, was also impacted by E2, we examined expression of *shh* and its targets: s*hh* was unaltered, while decreases in *patched2* (*ptch2)* and *GLI-Kruppel family member 1* (*gli1)* were observed by qPCR (**Figure 2.15 B-C;** *p<0.05, **p<0.01), although no changes in muscle or somite development were noted. To determine if E2-mediated HH pathway regulation caused *VEGFAa*, loss, we treated embryos with the smoothened agonist SAG. SAG enhanced *ptch2* expression and blunted the effect of E2. SAG likewise enhanced expression of *VEGFAa* in controls; however, it had no impact on *VEGFAa* expression in E2-treated embryos, indicating E2 antagonizes *VEGFAa* independently of HH (**Figure 2.15 D**). Together, these data suggest E2-mediated alterations in hemogenic niche specification are caused by loss of *VEGFAa* expression and downstream activity.

Figure 2.15

A. Arterial expression of *tbx20*, which was decreased by E2 exposure, could be rescued by increased *VEGFAa* levels, but not by NICD induction (n≥25).
B. Expression of *sonic hedgehog (shh)*, nor the development of HH-dependent tissues such as the somites, were altered by E2 as assessed by *myf5:GFP* and *myoD* expression.
C. qPCR revealed small, but significant, decreases in HH-responsive genes from E2 exposure (mean of triplicate experiments +/- SEM, one-tailed t-test *$p<0.05$; **$p<0.01$).
D. Loss of *ptch2* expression following E2 treatment could be alleviated by co-treatment with the smoothened agonist SAG (10 uM); in contrast, dual treatment with SAG could not rescue the E2-mediated loss of *VEGFAa* (n≥35).

Figure 2.15 (Continued)



Excess Estrogen Impairs HSC Development in the Mouse

To analyze conservation of the role of estrogen in specification of hemogenic endothelium and HSC emergence in a mammalian system, mice that lack the enzyme 5α-Reductase (*Srd*), which catalyzes the breakdown of testosterone into di-hydroxytestosterone were utilized. Progeny of *srd*-/- female mice are exposed to 2-3 fold elevated estrogen levels throughout gestation and display increased risk of hemorrhaging and death due to these high estrogen levels (Mahendroo et al., 1997). Use of multiple mating schemes revealed that the phenotype of the mother (+/-), rather than the pup, dictated the results obtained in the embryo. Upon removal of the uterus, *srd* mice were observed to have hemorrhaging throughout the uterine horn (**Figure 2.16 A**); blood clots were also noted at the placental junction of both viable and inviable implantations (**Figure 2.16 B**). Analysis of immunohistochemistry of the HSC marker c -kit and the endothelial marker PECAM (CD31) revealed decreased expression of each marker in the AGM and fetal liver of embryos exposed to high maternal estrogen levels compared to stage-matched WT controls (**Figure 2.16 C-D**). Interestingly, as the effects of elevated estrogen on HSC development appear to be largely due to the maternal genotype, not embryonic, it suggests that, as in zebrafish, the negative impact of elevated estrogen on HSC development in a mammalian species is due to maternally derived estrogen activity.

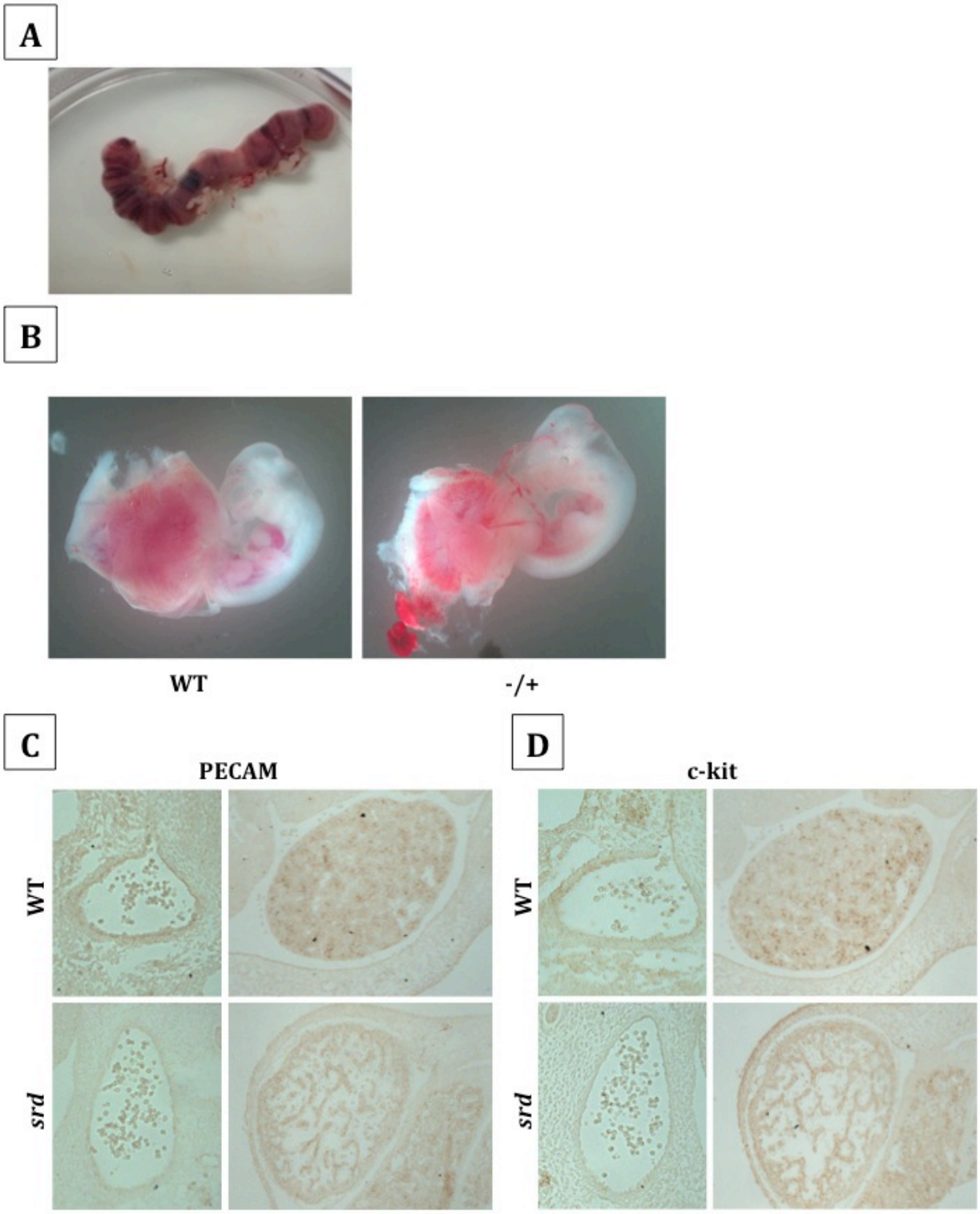
Xenoestrogens partially mimic the effects of 17β-Estradiol on HSCs

To determine if estrogens could limit HSPC production in the absence of deficiencies in maternal or fetal E2 regulation, we examined the impact of environmentally derived xenoestrogens. Three compounds, genistein (GEN), a component of soy products,

Figure 2.16

A. Mice from *srd* mothers displayed hemorrhaging during gestation. Embryos are shown at E11.5
B. *srd* mutant mice were largely normal but did display placental defects and blood clots.
C. PECAM staining was reduced in both the AGM (left) and fetal liver (right) of *srd* mice compared to WT at E11.5.
D. c-kit expression was decreased in the AGM (left) and fetal liver (right) of mutant mice at E11.5.

Figure 2.16 (Continued)



ethinylestradiol (EE), an estrogen found in hormonal contraceptives, and Bisphenol A (BPA), a hardening agent used in the manufacturing of plastics, were selected as representatives of the larger class of xenoestrogens. Upon xenoestrogen treatment, *runx1/cmyb* expression was reduced in all cases; which was confirmed by qPCR (**Figure 2.17 A,F**). Importantly, xenoestogen mediated HSPC loss could be reduced by treatment with FULV, indicating that the effects of each are mediated, at least in part, by signaling through esrs (**Figure 2.17 A**). GEN and EE treatment also partially replicated the vascular niche defects observed with E2: at the non-toxic doses utilized, no major abnormalities were seen in ISV formation by *flk1* staining (**Figure 2.17 B**), however arterial *Notch:GFP* was reduced by both GEN and EE (**Figure 2.17 C**). Exposure to GEN and EE likewise led to slight changes in the expression of arterial markers as well as *scl*, while BPA had no effect on these targets (**Figure 2.17 E-F**). Furthermore, a noticeable reduction in *VEGFAa* expression was caused by EE (**Figure 2.17 D**). While changes following xenoestrogen treatment were neither as severe nor as penetrant as those from excess E2, the same signaling cascades were impacted, suggesting exposure to environmental estrogens may have negative implications for HSPC specification.

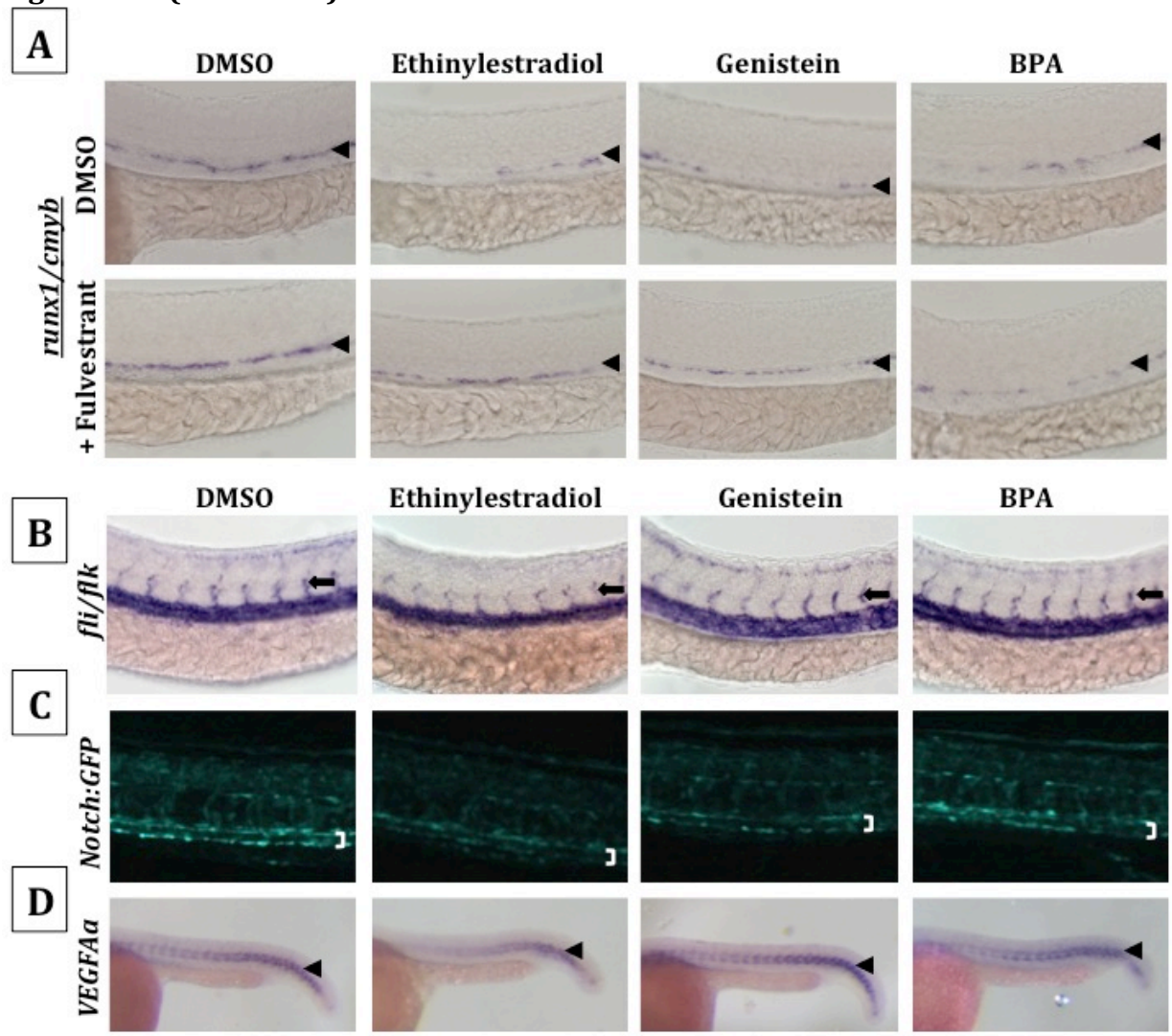
Antagonism of Estrogen Signaling Expands the Zone of VEGF Regulation

Having determined exogenous E2 and related estrogenic molecules can negatively impact HSC development through inhibition of the VEGF/Notch cascade and subsequent formation of hemogenic endothelium within the aortic vasculature, we next wanted to understand whether intrinsic levels of E2 played a functional role in niche specification. In contrast to excess E2, when embryos were exposed to the pan-Esr antagonist ZK, somitic expression of

Figure 2.17

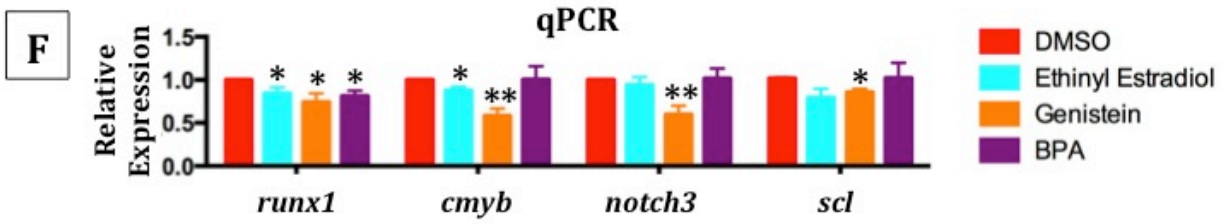
A. Exposure to xenoestrogens ethinyl estradiol (EE; 6μM), genistein (GEN; 7.5μM), or Bisphenol A (BPA; 50μM) decreased *runx1*/*cmyb* expression, and could be partially blocked by co-treatment with fulvestrant (n≥25/treatment).
B. Treatment with GEN reduced the expression of *fli/flk* in the intersomitic vessels (n≥25/treatment); arrow points to ISVs.
C. Treatment of GEN (15/25) and EE (12/23) decreased *Notch:GFP* expression.
D. *VEGFAa* was noticeably decreased by EE (15/26); arrowheads indicate somite region.
E. Summary of changes in the expression of vascular identify markers *ephrinB2*, *flt4, notch3,* and *deltaC* following treatment with xenoestrogens (n≥25).
F. qPCR confirmed decreases in *runx1*, *cmyb notch3,* and *scl* by xenoestrogen exposure (mean of triplicate experiments +/- SEM; one-tailed t-test *$p<0.05$; ** $p<0.01$).

Figure 2.17 (Continued)



E

	ephrin B2	%	*flt4*	%	*notch3*	%	*deltaC*	%
Ethinyl Estradiol	↓16/37	43	↑11/34	32	↓11/32	34	↓9/34	26
Genistein	↓22/34	65	↑10/30	33	↓12/37	32	↓5/35	14
BPA	↓3/35	8	↑3/37	8	↓4/38	10	No effect	-



VEGFAa was elevated at 24 hpf (**Figure 2.18 A);** downstream Notch:GFP activity was also enhanced by ZK (**Figure 2.18 E**). In other species, both Notch and VEGF family members contain functional EREs (Applanat et al., 2008; Soares et al., 2004), however, no consensus EREs were observed up to 8KB from the zebrafish *VEGF* transcriptional start site (*data not shown*). To confirm nascent E2 could nevertheless regulate VEGF activation, we modulated E2 activity in embryos expressing GFP under the regulation of 1.2kb of the *VEGF* promoter (He and Chen, 2005) (**Figure 2.18 B**); E2 strongly reduced *VEGF* activation, while ZK broadened the zone of expression. To determine if VEGF activity was also impacted, known transcriptional targets *dll4*, *dusp5* and *notch1b* (Bellou et al., 2009; Liu et al., 2003) (**Figure 2.18 6 C-D and F**) were examined: inhibition of E2-signaling by ZK strongly enhanced expression of each VEGF target compared to controls, suggesting VEGF-mediated transcriptional regulation is actively antagonized by nascent E2 during hemato-vascular development. Consistent with that finding, ZK treated embryos exhibited phenotypes reminiscent of those observed with *VEGF* overexpression in the ventral aspect of the AGM, including widening of the major vessels and ectopic or disorganized ISV sprouting (**Figure 2.18 G-H**) (Lawson et al., 2002; Wiley et al., 2011).

As shown above, zebrafish embryos contain endogenous E2 in the yolk, which is spatially positioned in a location proximal and just ventral to that of the developing AGM. To determine if antagonism of VEGF activity by nascent E2 impacted dorsal/ventral patterning of trunk vasculature, we next examined the boundaries of artery-vein specification following Esr antagonism. Whereas *ephrinB2* expression within the *fli1+* endothelial population was reduced by exogenous E2, it was noticeably enhanced by ZK treatment

Figure 2.18

A. *VEGFAa* expression was enhanced after inhibition of endogenous estrogen activity via the pan-Esr antagonist ZK164015 (18/32) as determined by WISH.
B. *gfp* expression was reduced in *VEGF:GFP* reporter embryos by E2 and enhanced by ZK (n>25).
C. The VEGF target gene *dll4* was decreased after E2 exposure but increased by ZK (n>20).
D. *dusp5* levels were reduced by E2 treatment but elevated after Esr antagonism (n>20).
E. Notch activity was elevated (12/19) and expression observed in the vein after treatment with ZK in a subset of embryos (5/19).
F. Increased expression of *notch1b* in the artery and ISVs was observed after ZK treatment (n>20).
G. Aberrant development (excess sprouting) of the intersomitic vessels in *flk1:GFP* transgenic fish was observed after treatment with ZK164015 (n≥22).
H. The diameter of the axial vessels as assessed by *fli1/flk1* was greater following inhibition of estrogen signaling by ZK than in controls (n≥25).

Figure 2.18 (Continued)

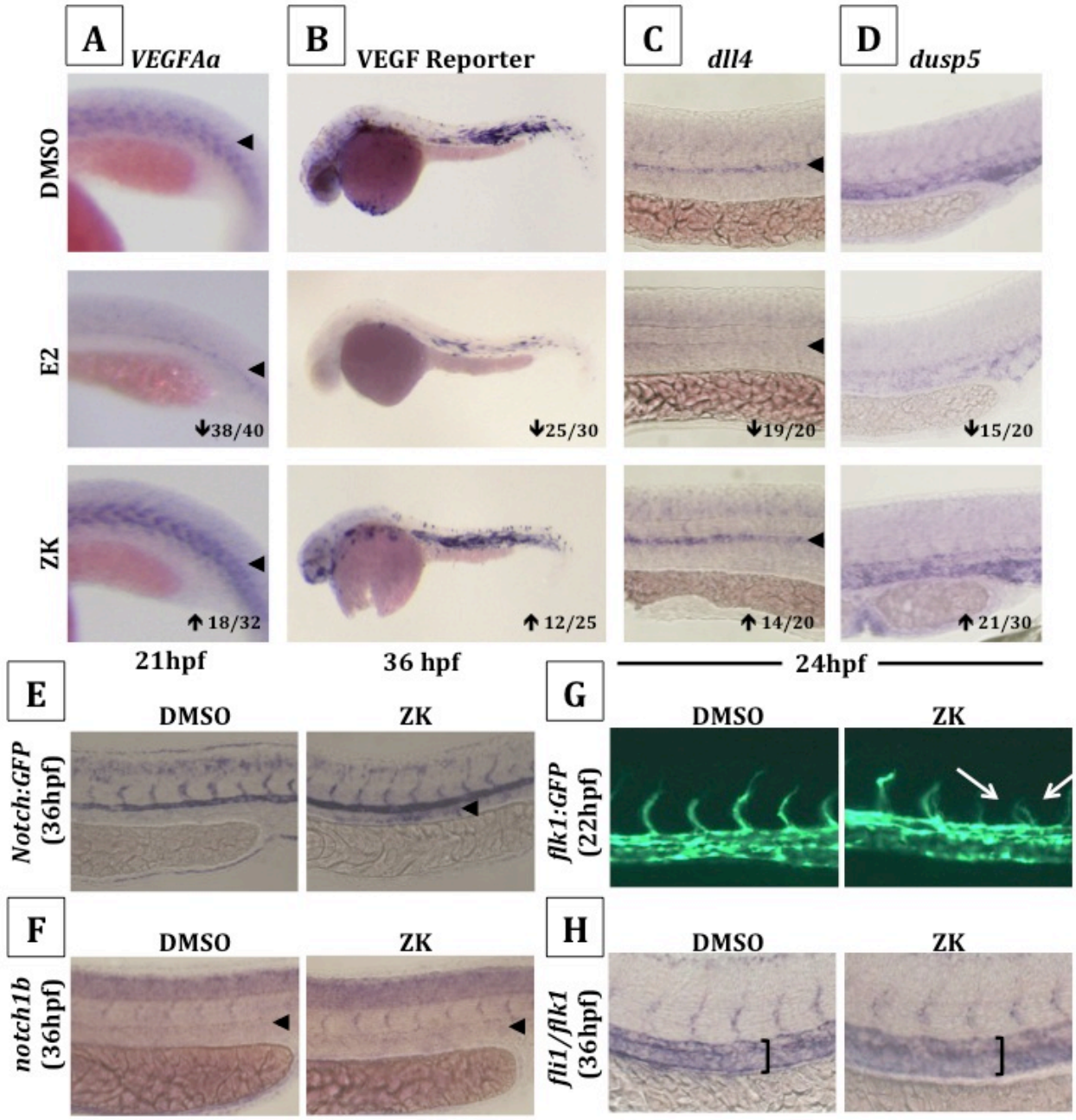
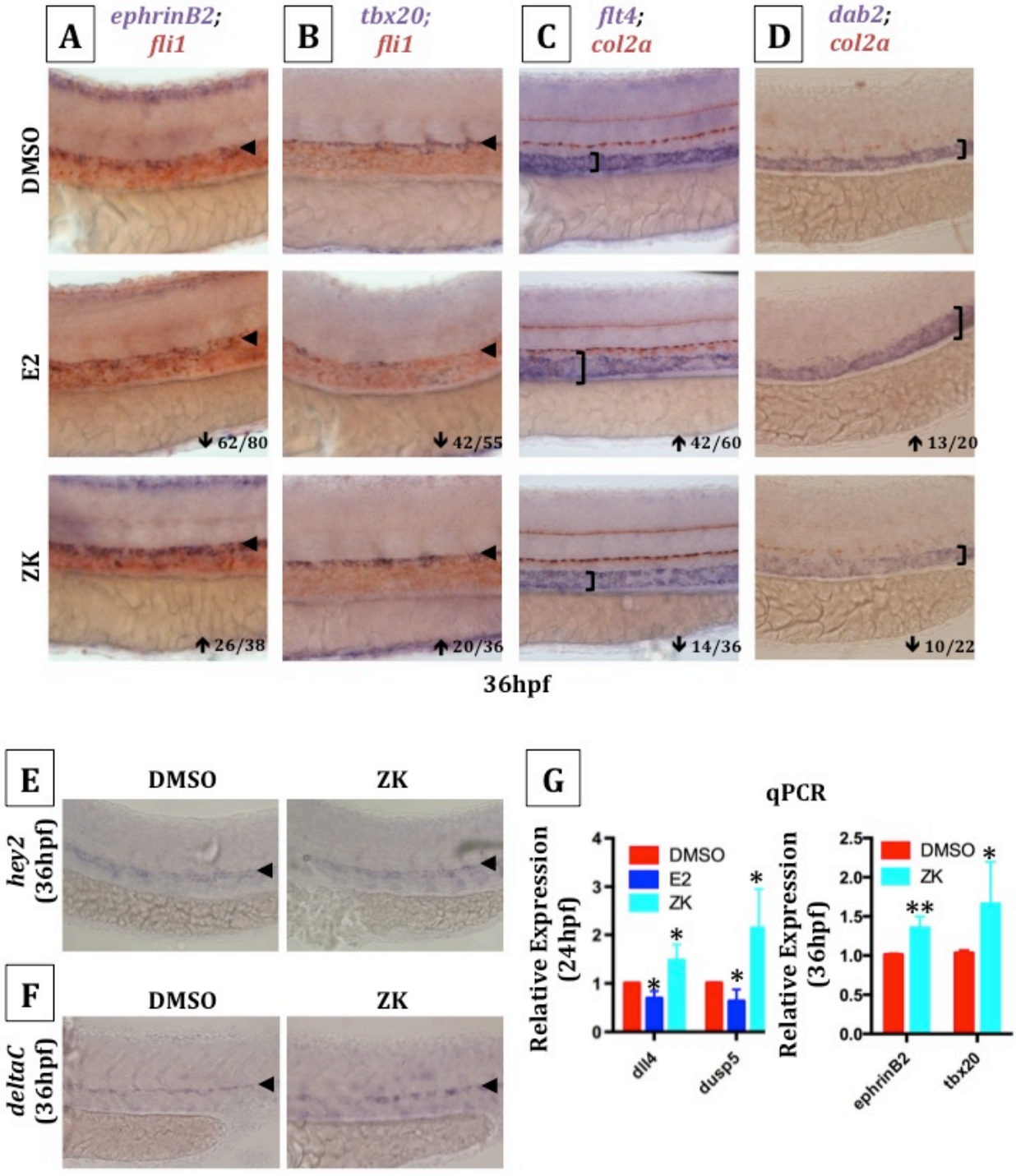


Figure 2.19

A. Arterial *ephrinB2* expression in the *fli1+* vasculature was enhanced following inhibition of estrogen signaling by ZK (26/38).
B. *tbx20* expression in the roof of the aorta was substantially increased by ZK treatment (20/36).
C. Venous *flt4* was expanded dorsally toward the *col2a+* hypocord by E2 but was reduced in expression by ZK exposure (14/36).
D. The venous marker *dab2* was similarly regulated as determined by WISH (n>20).
E. *hey2* expression was similarly enhanced after exposure to the Esr antagonist ZK (n>20).
F. *deltaC* expression was increased following antagonism of Esr signaling (17/38).
G. qPCR analysis confirmed changes in VEGF-responsive and arterial gene expression (mean of triplicate experiments +/- SEM, one-tailed t-test *$p<0.05$; **$p<0.01$).

Figure 2.19 (Continued)



(**Figure 2.19 A**); similar results were observed with *deltaC* and the Notch target *hey2* (**Figure 2.19 E-F**). Expression of VEGF-regulated *tbx20* was likewise increased by ZK, including expansion to both walls of the aorta from its characteristic position on the roof (Wilkinson et al., 2009) (**Figure 2.19 B**); enhanced expression of arterial markers was confirmed by qPCR (**Figure 2.19 G**; *p<0.05, **p<0.01). In contrast, while the dorsal limit of venous *flt4* was heightened by E2 toward the *col2a+* hypochord, within the arterial space, expression was diminished and patchy in embryos exposed to ZK (**Figure 2.19 C**). Identical findings were observed for *dab2*, where embryos showed reduced expression in the vein after ZK treatment (**Figure 2.19 D**), indicating the zone of VEGF-regulation was affected by modulation of estrogen activity.

Loss OF E2-Mediated VEGF Regulation Alters Hemogenic Potential of the Vein

Hemogenic endothelium is normally situated within arteries, such as the dorsal aorta; we next sought to determine if loss of E2-mediated antagonism of VEGF activity would be sufficient to convert the functional potential of venous endothelium. Expression of the hemogenic endothelial marker *scl* was elevated in ZK treated embryos, including induction in the vein in a subset of embryos (**Figure 2.21 A**) This result was confirmed using *scl:GFP/flk1:dsRed* embryos where ZK exposure increased the number of double positive vascular cells (**Figure 2.20 A).** *runx1* expression in the artery was likewise increased via ZK as determined by both WISH and qPCR (**Figure 2.20 B,D**; *p<0.05) and could be observed in the vein in a subset of embryos. This phenotype, reminiscent of that previously reported for Notch overexpression (Burns, 2005), was also found after genetic suppression of *esr2* signaling via MO knockdown (**Figure 2.21 B**). While reassignment of arterial fate as

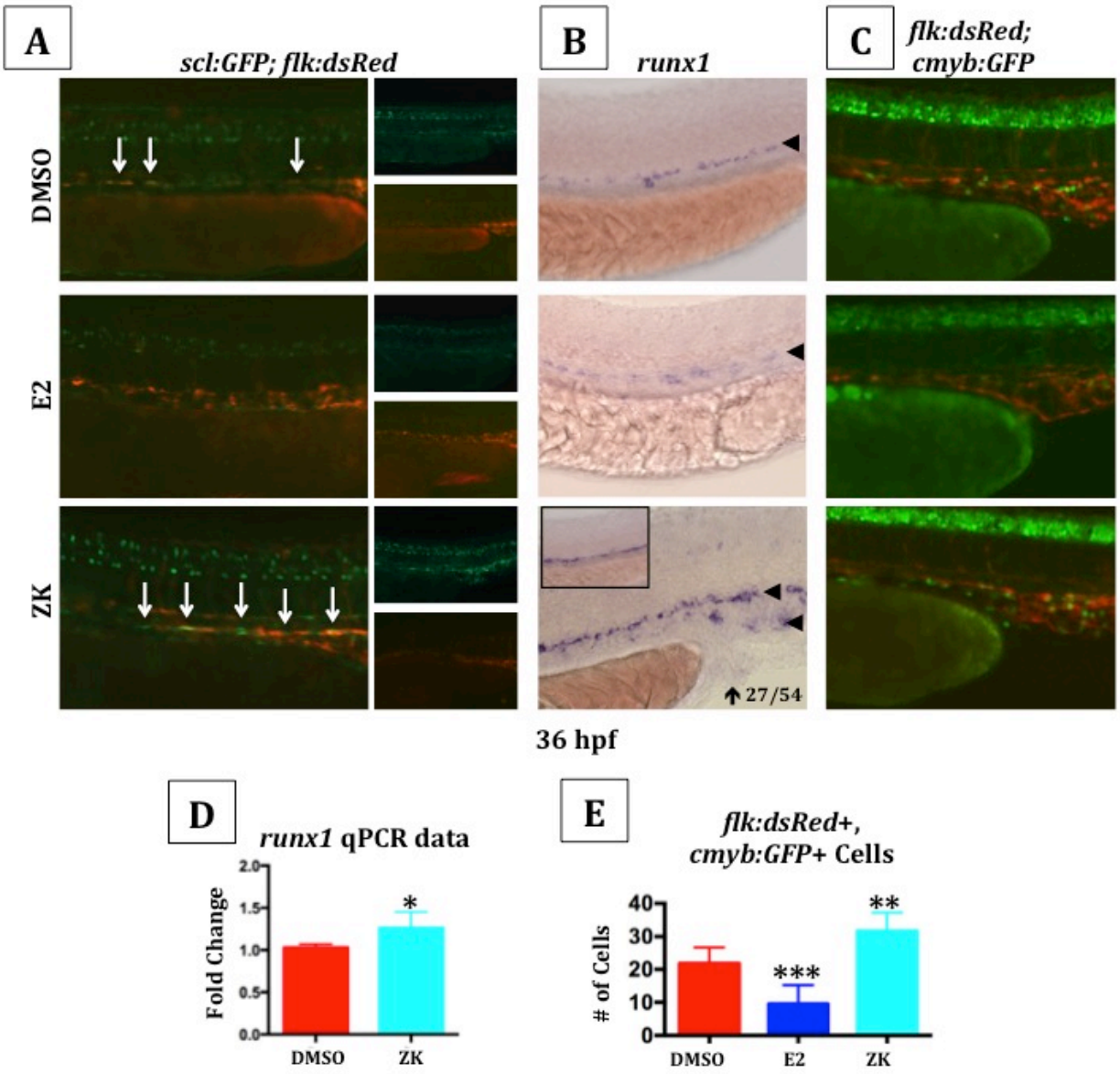


Figure 2.20

A. Hemogenic endothelium, as assessed by the dual expression of *scl:GFP* and *flk:dsRed*, was reduced by exogenous E2 treatment but enhanced following Esr antagonism by ZK (n>15).
B. *runx1* expression, as seen by WISH, increased (27/54) after estrogen receptor inhibition, with mislocalization to the vein (10/54).
C. Expression of *flk:dsRed+; cmyb:GFP+* HSCs was enhanced by treatment with ZK (n≥10).
D. Quantification of changes in *runx1* expression (mean of triplicate experiments +/- SEM; one-tailed t-test *p<0.05).
E. The number of *flk:dsRed* and *cmyb:GFP+* dual positive HSCs was decreased by E2 and enhanced after treatment with ZK (one-tailed t-test **p<0.01, ***p<0.001).

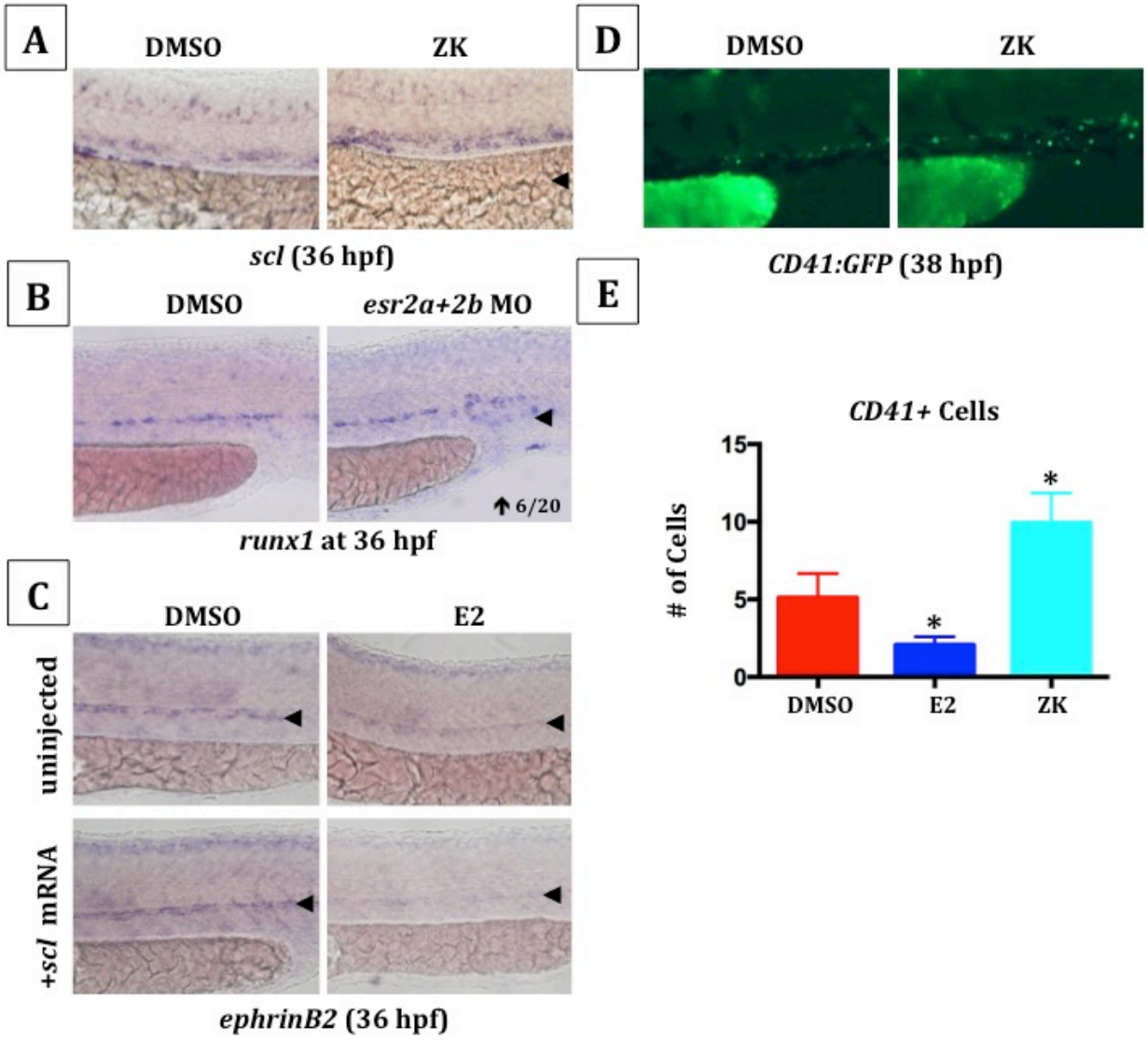


Figure 2.21

A. Expression of *scl* was increased after ZK exposure (12/20) with occasional induction in the venous vasculature (5/20).
B. *runx1* expression was noted in the vein after combined MO knockdown of *esr2a+b* (6/20).
C. *ephrinB2* expression was not rescued after injection of *scl* mRNA into E2 treated fish (n>40).
D. *CD41:GFP*+ cells increased when E2 signaling was antagonized by ZK treatment (n≥13).
E. Quantification of the number of *CD41+* cells after E2 treatment and antagonism (one-tailed t-test *p<0.05).

marked by *ephrinB2* (**Figure 2.19 A**) was not found after ZK treatment, the percentage of embryos with venous *runx1* expression was proportional to those exhibiting Notch:GFP in the vein (**Figure 2.18 E**); this finding was also congruent with *scl* mRNA-mediated recovery of HSPCs following E2 exposure, where *runx1* expression was restored **(Figure 2.10 H)** in the absence of *ephrinB2* rescue (**Figure 2.21 C**). To confirm alterations in E2-signaling had functional consequences for HSC production, we assessed HSC content using *CD41:GFP+* and *flk:dsRed+/cmyb:GFP+,* previously shown to contain functional HSCs by embryo-to-adult transplantation (Harris et al., 2013) and *lineage tracing* (Bertrand et al., 2010), respectively. Following ZK exposure, the number of mature *CD41:GFP*+ HSCs in the AGM region was significantly increased **(Figure 2.21 D-E**; $p<0.05$). Analysis of double *flk1:dsRed+/cmyb:GFP+* cells budding from the trunk vasculature was similarly elevated **(Figure 2.20 C, E)**. Finally, to demonstrate that the increase in HSPCs mediated by ZK was due to elevated VEGF activity, we utilized previously described VEGF pathway mutants: *kdrl*^{y17} embryos exhibit normal vasculogenesis but defective VEGF-mediated ISV sprouting, whereas *plcg*^{y13} mutants show no ISV production, indicative of a block of increasing severity in VEGF activity (Covassin et al., 2009). Control and ZK-treated embryos were sorted based on *fli1:GFP+* ISV expression **(Figure 2.22 C)**; *kdrl*^{y17} mutants displayed reduced *runx1* expression, however, exposure to ZK boosted HSPC number as determined by WISH **(Figure 2.22 A)**. In contrast, whereas *plcg*^{y13} mutants also displayed a virtual absence of *runx1+* expression, the block in HSPC formation could not be alleviated by ZK exposure **(Figure 2.22 B),** indicating VEGF activity was the target of E2 regulation. Together, these data lead to a model (**Figure 2.23**) whereby maternally deposited E2 functions to pattern the HSC niche via antagonism of the ventral limit of VEGF regulation,

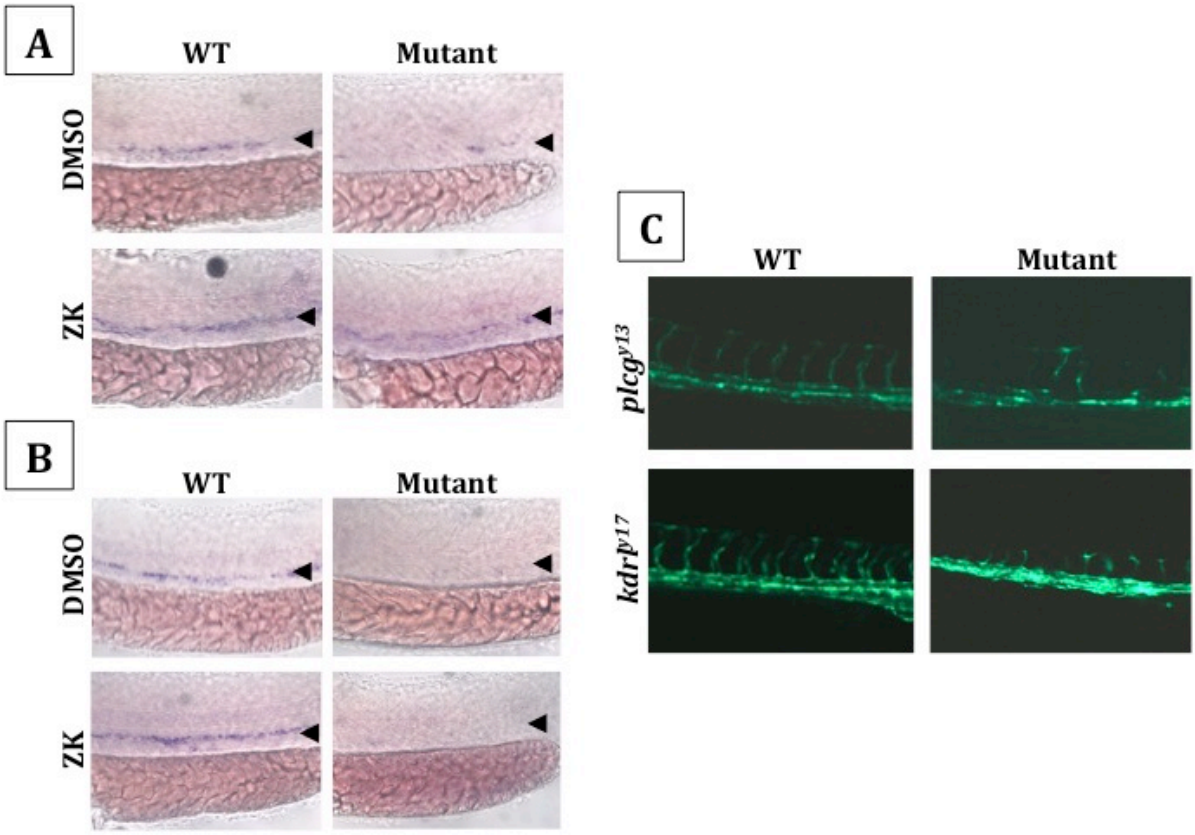


Figure 2.22

A. WISH analysis in *kdrl*^{y17} mutant embryos indicate loss of *runx1*, which can be partially restored by reduction of E2-mediated VEGF antagonism after ZK treatment (n>20/condition).
B. WISH analysis in *plcg*^{y13} mutant embryos, which have significant loss of VEGF activity, show complete loss of *runx1* expression in the presence or absence of ZK (n>20/condition).
C. Alterations in the expression of *fli:GFP*+ intersomitic vessels were noted in both *plcg* (severe) and *kdrl* (moderate) mutant embryos compared to sibling controls (n≥20).

allowing for the "appropriate" assignment of the hemogenic endothelial niche and subsequent HSPC production.

Discussion

In this paper, we identify estrogens as previously uncharacterized regulators of embryonic hemato-vascular niche patterning. Estrogens are considered the most ancient of the steroid hormones and have a remarkable ability to act as morphogens, signaling molecules and transcription factors simultaneously, thus controlling a myriad of downstream processes through a combination of their relative concentration, receptor distribution and availability of direct genetic targets (Heldring et al., 2007; Thornton, 2001). We propose that in zebrafish embryos (**Figure 2.23,** *top panel*), endogenous E2 maternally deposited in the yolk diffuses to define the ventral boundary of somite-derived VEGF regulation. The opposing dorsal-ventral gradients result in the artery receiving higher VEGF and lower E2 relative to the vein, and enables "proper" vasculature patterning with coincident localization of the hemogenic endothelial niche to the artery. When embryos are exposed to excess levels of E2 (**Figure 2.23,** *middle panel*), or related xenoestrogens, the gradient is disrupted and additional estrogen is present to antagonize the VEGF/Notch cascade in all cell types expressing Esrs. In this scenario, the total region of VEGF (and Notch) activity decreases leading to loss of arterial markers in the developing vasculature, with a concomitant enhancement in venous identity, and reductions in hemogenic endothelial specification. In contrast, when Esr signaling is inhibited (**Figure 2.23,** *lower panel*), the functional impact of the gradient of E2 from the yolk is lessened, allowing the domain of

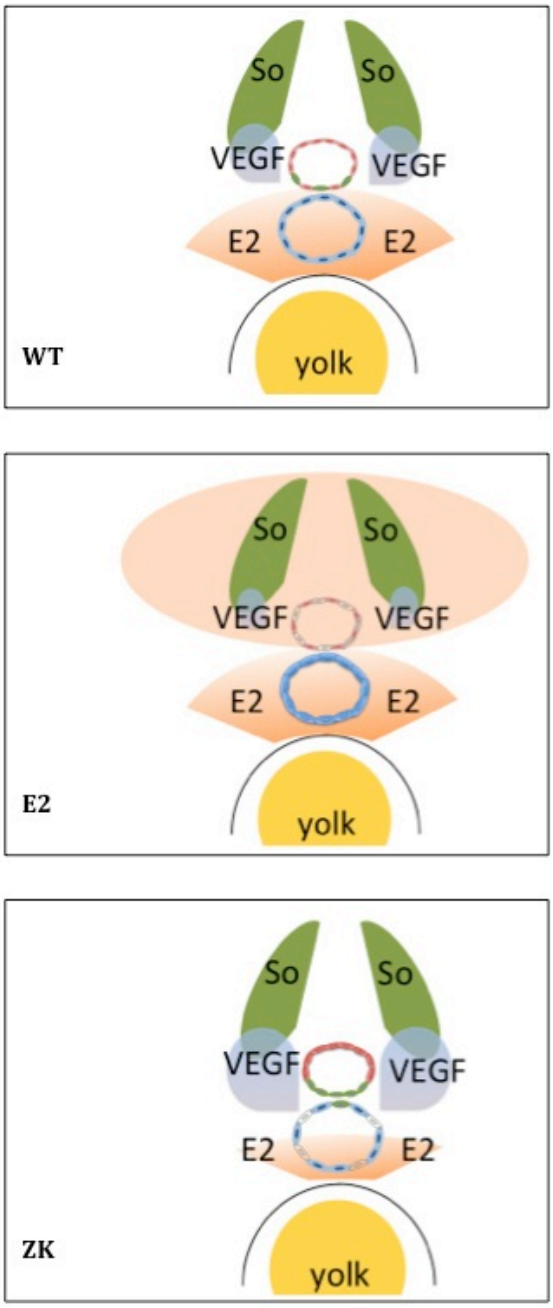


Figure 2.23

Model: in WT embryos (*upper panel*), opposing gradients of E2 and VEGF specify the arterial/venous boundaries of hemogenic endothelium and subsequent emergence of HSPCs (red circle = artery; blue circle = vein). Excess E2 (*middle*), disrupts this gradient, further antagonizing VEGF and causing artery, hemogenic endothelium and HSPCs to fail to form properly. In contrast, inhibition of esr activity (*lower panel*) allows of the range of VEGF regulation to increase causing ectopic hemogenic endothelial specification in the vein.

VEGF regulation to increase: expression of VEGF targets- including the notch pathway- are enhanced, repression of venous markers is observed, and genes required for hemogenic endothelial specification are elevated, jointly causing occasional mislocalized production of *runx1+* HSPCs in the vein independent of arterial reassignment (**Figure 2.20 B**). This study highlights the ability of E2 to act simultaneously as a morphogen, signaling molecule and (presumably) transcription factor to pattern an embryonic niche outside of the urogenital system, and further, in its novel role in setting the limits of VEGF regulation, may help resolve the discrepancies between the necessity of arterial gene expression and hemogenic HSPC production.

Fine tuned regulation of estrogen availability during early development is highly conserved in placental mammals: maternal estrogen levels are quite low early in pregnancy but steadily increase until delivery (Tulchinsky et al., 1972). Our data suggest that E2 levels may be actively suppressed during early embryonic development in part to avoid negative impacts on vasculature specification and maturation, including hemogenic endothelium localization. While there are clear anatomical differences between yolk-supported and placental vertebrate embryos, recent studies have indicated that the gene networks and cellular movements establishing the fate of vascular progenitors is VEGF-concentration dependent (Kohli et al., 2013) and more similar across species than previously appreciated (Lindskog et al., 2014). Beyond reports of myeloproliferative disorders in a subset of adults (Shim et al., 2003), few hematopoietic phenotypes have been noted, thus far, in mice with homozygous *Esr1* or *Esr2* mutations (Krege et al., 1998; Lubahn et al., 1993); this finding is consistent with our data indicating early *esr2* knockdown enhances, rather than negatively

impacts, HSC production. Likewise, our observations concerning exposure to exogenous E2 are reminiscent of prior reports of placental insufficiency, intrauterine hemorrhaging and eventual death observed in embryos of *Steroid 5α-Reductase* deficient dams, which have maternally-derived elevations in E2 (Mahendroo et al., 1997). As our preliminary work suggests that HSC development is impaired in these mice; it will be interesting to examine more closely how E2 modulation impacts hemogenic endothelial specification and HSC induction in mammalian models.

In zebrafish, we can attribute the negative impact of exogenous E2 on HSC formation to repression of VEGF-Notch signaling during the window of hemogenic endothelium development. While E2 has long been recognized as having the ability to modulate both VEGF and NOTCH expression in other contexts, its impact on the development of the hemato-vascular system during embryogenesis is novel. Indeed, while ERE sites in *NOTCH* and *VEGF* are functional in mammals, most evidence from breast cancer studies has suggested that E2 acts to induce both pathways rather than to repress them, as we observed (Buteau-Lozano et al., 2002; Soares et al., 2004). Notably, however, E2 has been reported to antagonize VEGF signaling in hypoxic conditions (Miyamoto et al., 2002). While we found no evidence of a consensus ERE in the *VEGF* promoter up to 8kb from the start site (*data not shown*), increasing evidence suggests that the hematopoietic niche is a hypoxic environment in the embryo and adult, and we have recently shown that Hypoxia Inducible Factor 1 alpha (HIF1α) is a critical regulator of hematopoietic development (Harris et al., 2013); additional studies will be needed to determine whether hypoxia and/or the HIF1 complex are interacting with E2 in this context to directly or indirectly

impact *VEGF* production and downstream function. Our findings on the timing of E2-mediated hemogenic niche regulation indirectly support the hypoxia model, as the negative impact of E2 on VEGF is only observed prior to the start of circulation and maturation of oxygen-carrying erythrocytes beginning at 24hpf. It will be important to determine if transitioning out of this relatively hypoxic state post-circulation onset is the reason E2 displays differential effects in later hemato-vascular development. Also of potential consequence to the findings reported here, recent investigations in Xenopus indicate different VEGF isoforms are required for the establishment of arterial versus hemogenic fate during development (Leung et al., 2013); while it has not yet been demonstrated whether this will also be the case in zebrafish or mammalian embryos, it will be valuable to determine if estrogens could play a role in transcript selectivity.

Finally, the observation that common xenoestrogens such as genistein and ethinylestradiol can similarly impact vascular patterning and the subsequent specification of HSPCs suggests environmentally relevant estrogenic substances may have unexpected impact on hemato-vascular development *in vivo*. Although the xenoestrogens tested were not as potent as E2, as a group they are present at increasingly elevated concentrations in our environment; the specific representative examples tested may have had difficulty activating zebrafish Esrs at a sufficient level to elicit a major response, as differences in receptor affinity compared with endogenous estrogens have been previously described (Barkhem et al., 1998). The fact that xenoestrogens could affect the HSC niche, raises important questions about the long-term deleterious effects of *in utero* exposure on hematopoietic development and homeostasis. While not specifically examined here,

pregnant mice fed a diet rich in genistein had pups with increases in erythropoiesis and granulopoiesis at 12 weeks of age (Vanhees et al., 2011). Genistein can induce rearrangements in the Mixed Lineage Leukemia (MLL) gene in human CD34+ cells that resembled those commonly seen in infant leukemias (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007) and exposure to dietary flavonoids during pregnancy has been linked to the development of infant leukemias through epidemiological studies (Ross, 2000). Our data indicate that concentration and timing significantly alters the outcome of exposure to estrogenic compounds, and despite negative effects on hemogenic niche specification, exposure at slightly later stages of development produces strikingly different results, including increasing the proliferative capacity of newly produced HSCs. Whether this could have long-term consequences like those associated with xenoestrogen exposure still needs to be determined. However, there is substantial evidence that hematopoietic cells continue to be susceptible to estrogenic regulation. During gestation, maternal blood volume grows by ~50% to accommodate the growing oxygenation and nutritional needs of the fetus (Hytten, 1985), with recent investigations indicating this is due, at least in part, to E2-mediated enhancement in the proliferation of HSPCs (Nakada et al., 2014). Further, when guinea pigs were administered exogenous E2, blood volume increased to levels comparable to that seen during pregnancy, indicating it is a critical regulator of hematopoietic output (Davis et al., 1989). In contrast, in rats, reduced E2 levels as a result of ovariectomy led to hematopoietic dysfunction, characterized by increased extramedullary hematopoiesis (Qiu et al., 2012). However, exposure to excess E2 has also been associated with reductions in HSC number (Perry et al., 2000). Together with our data, these studies indicate E2 may regulate of several aspects of hematopoiesis and suggest

spatio-temporal alterations in the concentration of this previously underappreciated, but well conserved, molecule will have significant impact beyond embryogenesis. Here, we specifically demonstrate maternally derived E2 has a central role in establishing dorsal-ventral patterning of the hemato-vascular system in zebrafish, acting as a ventral limiting factor to specify the hemogenic endothelial niche.

Experimental Procedures/Materials and Methods

Zebrafish Husbandry

Zebrafish were maintained according to BIDMC IACUC protocols. Zebrafish lines utilized were previously described and are listed in **Appendix II.**

Chemical Treatments and Evaluation

Embryonic zebrafish exposures were performed from 5 somites until 36hpf in multiwell plates, unless otherwise specified. The following compounds (Cayman Chemicals unless otherwise indicated) were used: 17β-estradiol (E2: 8-10 μM); 17α-estradiol (10 μM); 2,3-*bis*(4-hydroxyphenyl)propionitrile (DPN:10 μM); propylpyrazole triol (PPT: 20 μM); fulvestrant (FULV (ICI-182,780): 15 μM); genistein (GEN: 7.5 μM); ethinyl estradiol (EE: 6 μM); bisphenol A (BPA:30 μM (Sigma)); DAPT (GSI-IX) 20 μM); G1 (1 μM); G15 (1 μM); ZK164015 (ZK:10 μM (Tocris)); SAG (10 μM); SU1498 (10 μM (Calbiochem)) and screen hits (10 μM). Whole mount in situ hybridization (WISH) was performed using previously published probes and methods (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). Qualitative phenotypes (≥3 replicate clutches) are scored as the # altered/# analyzed per treatment. *plcg1*$^{y13/y13}$ and *kdrl*$^{y17/y17}$ mutants were identified post-treatment and

evaluation by genotyping as previously described (Covassin et al., 2009; 2006). Fluorescence Activated Cell Sorting (FACS) analysis of transgenic lines (pools of 3 embryos x >10 biological replicates), phospho-Histone H3 (pHH3) labeling (Abcam) and 5-bromo-2'-deoxyuridine (BrdU) incorporation (Sigma) were performed as previously described (Harris et al., 2013; North et al., 2007). Images were acquired using a Zeiss Axio Imager A1 or Zeiss Discovery V8/Axio Cam MRC and Axiovision LE software (Carl Zeiss, Oberkochen, Germany).

Morpholino (MO) and mRNA Injections

MOs (**Appendix II;** Gene Tools) were injected into one cell stage embryos and treated and analyzed as detailed above; the *ERE:GFP* line was used to confirm specificity. *scl* mRNA was synthesized using manufacturer's directions for Ambion mMessage mMachine as previously described (Kim et al., 2013) and injected (150 pg) into one-cell stage embryos. Treatments and analysis were performed as described above.

Quantitative RT-PCR

cDNA was isolated from pooled (n=30) embryos at 24 or 36hpf and isolated using an RNAqueous kit (Ambion). qPCR reactions (primers listed in **Appendix II**) were performed using an ABI 7900 machine and analyzed as previously described (North et al., 2007) using the ddCt method with either *18s* or *tbp* as the reference gene

Heatshock Modulation of Notch and VEGF signaling

Embryos were treated with test compounds prior to and post-heat shock as indicated. *hs:VEGFAa* and *hs:Gal4 x UAS:NICD* expression was induced by incubation in a 38°C circulating water bath for 10 or 7 minutes, respectively, at ~16hpf. *hs:VEGFAa* fish were identified by cardiac GFP expression and *hs:Gal4 x UAS:NICD* fish by genotyping post-WISH analysis.

Estradiol Immunoassay

E2 content was measured using an Estradiol EIA Kit (Cayman Chemical). Embryos (n=30) were pooled and homogenized in 100uL of EIA buffer and diluted to 300uL total volume; 50uL was used per reaction according to manufacturers instructions. For yolk content analysis: the yolk was manually removed by pipetting (10uL tip) and embryos were pelleted for 30sec at 300 rpm; yolk extract (supernatant) was collected, and the pellet (body) disaggregated and resuspended in 300 uL EIA buffer as above. Analysis was performed according to instructions from Cayman Chemical.

AGM/Fetal Liver Histology

Timed matings were used to stage embryos. Embryos were removed from the uterus at E11.5 for processing by histological evaluation. Hematoxylin and Eosin was used to stain serial paraffin sections. Ckit (eBiosceince) and PECAM (eBioscience) antibodies were also used on serial sections of the AGM and fetal liver.

Chapter 3:
Estrogen Enhances the Number of Hematopoietic Progenitors during Vertebrate Development and Marrow Regeneration

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Attributions

Estrogen signaling was identified as a modifier of *runx1/cmyb* expression by WG and TEN. KJC and TEN performed zebrafish irradiation studies and FACS analysis, zebrafish development studies, murine erythrocyte work, proliferation analysis, and baseline male/female marrow analysis. MD helped with zebrafish irradiation work and embryonic erythrocyte studies. SL, GF, and VE assisted with kidney dissection and FACS preparation. WG and TEN performed the CFU-S12 assay. KJC, MD, WG, and TEN designed experiments and evaluated results.

Introduction

After niche specification, exposure to exogenous estrogen (E2) during embryogenesis elevated the expression of HSC markers (**Figure 2.9**) (KJC *in press*); this effect appears to be at least partially mediated by an estrogen-induced increase in cell cycling of the hematopoietic stem and progenitor population (HSPC). However, whether the adult hematopoietic system, including the HSPC population, is sensitive to hormonal modulation remains unclear. Likewise, it has not been prospectively established whether well-characterized differences in the baseline hematocrit between the sexes is due in total or in part to differential hormone regulation. Adult females have long been known to have a lower hemoglobin levels than males (Valberg et al., 1976), an observation that is conserved across many species (Murphy, 2014), and is suggestive of sex-specific hematopoietic regulation. Additionally, more then 75% of patients who suffer from autoimmune diseases are female, indicating potential hormone-mediated sex biases in hematopoietic function (Fairweather et al., 2008; Oliver and Silman, 2009). In contrast, men are known to be at a greater risk for developing acute leukemia (Jackson et al., 1999). In spite of these well-established gender differences in the function of the hematopoietic system, the mechanisms underlying these observations, and the putative role of hormones in these discrepancies, remain poorly understood.

Hormonal modulation been suggested for many years to impact erythropoiesis in adult animals; thyroid hormone (Popovic et al., 1977), testosterone (Zanjani and Banisadre, 1979) and estrogen (Landshman and Bleiberg, 1979), among other hormones (Fisher and Crook, 1962), have been investigated for their roles in erythropoiesis. Of these, testosterone has been of particular importance as it has been used clinically in the

treatment of anemia (Shahidi and Diamond, 1961). While estrogen is as a putative modifier of hematopoietic output due to its role in pregnancy-induced changes in blood volume (Davis et al., 1989), the means by which estrogen might be affecting hematopoietic homeostasis remain poorly understood. Outside the context of pregnancy, studies have indicated that endogenous E2 plays a role in HSC homeostasis and that excess E2 can enhance HSCs (Illing et al., 2012; Qiu et al., 2012). However, other work has suggested estrogen has a negative impact on hematopoietic maintenance (Li et al., 2013; Perry et al., 2000). Together, these studies suggest that hematopoietic homeostasis is highly sensitive to estrogenic regulation, although the mechanisms behind these somewhat contradictory observations remain poorly elucidated, as does a putative role for estrogen in hematopoietic regeneration.

Here, we demonstrate that the irradiated hematopoietic system of female zebrafish recovers faster from ablation than male fish, suggesting differential endogenous regenerative capacity between the sexes. We also find that treatment of zebrafish with excess estrogen can accelerate recovery of hematopoietic progenitors after injury in both sexes, while *ex vivo* treatment of murine bone marrow with estrogen led to increased numbers of spleen colonies in transplanted recipient mice. Elevation in hematopoietic precursors was also conserved in embryonic stages as zebrafish exposed to E2 during primitive erythropoiesis showed increased numbers of erythroid progenitors; however, E2 treated embryos possessed fewer functional erythrocytes, indicative of a block in maturation, and suggesting that E2 can regulate both hematopoietic progenitor number and function in addition to that of the HSC. This observation was likewise conserved in a murine system where exposure to excess maternal estrogen during gestation impaired

erythrocyte maturation and led to an enhanced propensity for embryonic lethality. Together, these results indicate that estrogen plays multiple roles in the homeostasis of the blood system and is likely a key regulator of Hematopoietic Stem and Progenitor Cell (HSPC) function.

Results

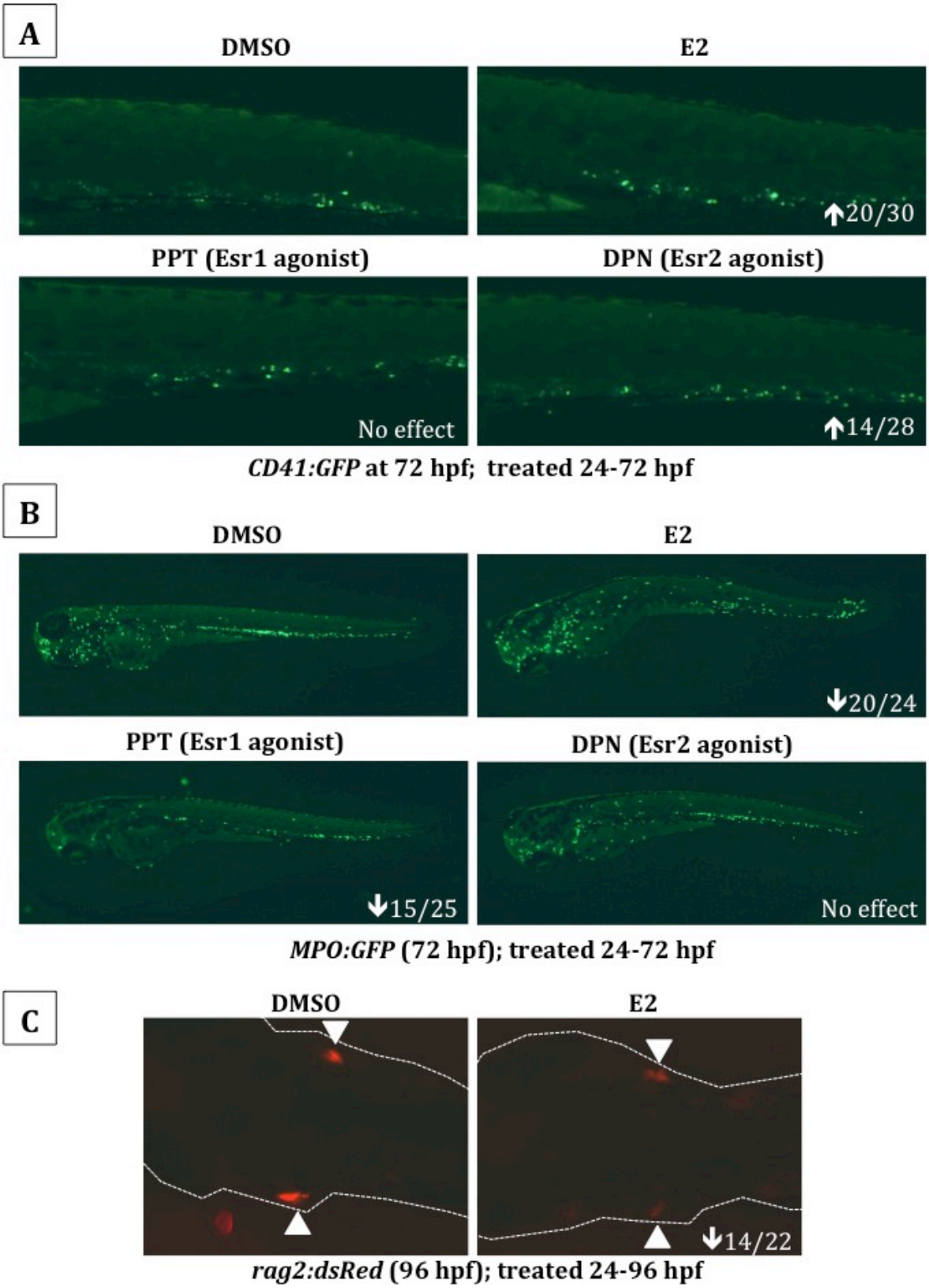
E2 Enhances HSC Formation during Development

As previously described, treatment of developing zebrafish with estrogen (E2) after specification of the hematopoietic niche, from 27-33 hpf during the window of HSC commitment and induction from the hemogenic endothelium, increased the expression of *runx1* and *cmyb* (**Figure 2.9**). We therefore sought to determine if the HSC continued to be responsive to E2 after this narrow window in development. When (*Tg(-6.0itga2b; (CD41):eGFP*)) (*CD41:GFP*) embryos, which mark HSCs, were exposed to E2 from 24-72 hpf, increased GFP positive cells were observed in the Caudal Hematopoietic Tissue (CHT), a secondary site of hematopoiesis thought to be the fetal liver equivalent (Murayama et al., 2006). This effect appeared to be mediated by Esr2, as more GFP+ cells were noted after treatment with the Esr2 agonist DPN, but not after exposure to the Esr1 agonist PPT (**Figure 3.1 A**). In spite of the increase in HSCs noted following exposure to excess E2, fewer cells were found in a myeloid GFP reporter line Tg*(mpx:GFP*) after treatment in the same window (**Figure 3.1 B**), suggesting that the hematopoietic stem and progenitor cells generated after exposure to exogenous E2 may have defects or a bias in their maturation potential. Interestingly, treatment with the Esr1 agonist PPT also reduced the number of *MPO+* cells while the Esr2 agonist DPN did not, indicating that the increase in HSCs and the

Figure 3.1

A. More *CD41+* cells were observed after treatment with E2 or DPN (n>25).
B. A reduction in the number of *MPO+* cells was found after treatment with E2 or PPT (n>20).
C. Fewer *rag2+* cells were noted after E2 treatment (n>20).

Figure 3.1 (Continued)



reduction in myeloid cells observed after E2 treatment from 24-72 hpf may not be mediated by the same estrogen receptor. Treatment of Tg*(rag2:dsRed2)* fish with E2 from 30-120 hpf suppressed the number of thymic lymphocytes (**Figure 3.1 C**), indicating lymphopoiesis is also impacted by E2 exposure. The dual blockage of lineage differentiation observed after E2 treatment suggests that, while E2 can enhance the appearance of phenotypic HSPCs in the embryo, it may simultaneously impair or bias their ability to mature into functional effector cells.

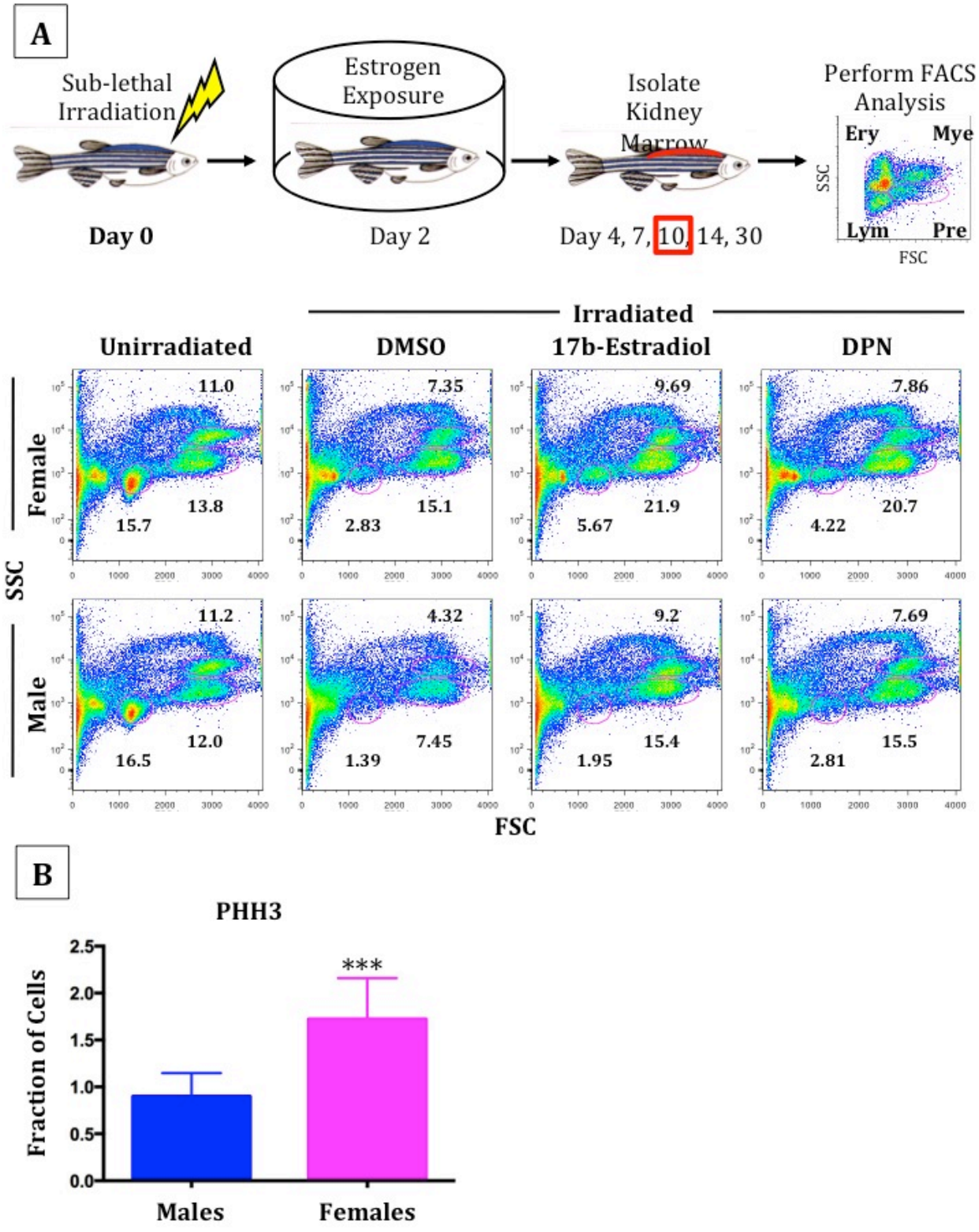
Estrogen Accelerates Hematopoietic Recovery After Irradiation Insult

As estrogen has the ability to increase HSC number during development, we next sought to determine if estrogen could also control HSC homeostasis in adult zebrafish after irradiation-mediated hematopoietic ablation. FACS Analysis of the kidney marrow (KM), the site of hematopoiesis in the adult zebrafish, by forward scatter- and side-scatter profiling enables resolution of the progenitor, lymphoid, and myeloid fractions of the marrow (Traver et al., 2003; 2004) and provides a method for assessing recovery of the hematopoietic system after irradiation insult. Interestingly, a sex bias was found in our results, whereby female fish had nearly twice as many progenitors as male fish 10 days post-irradiation, suggesting that females have enhanced regenerative capacity of the HSPC population compared to males (**Figure 3.2 A**). As hormone levels are known to be very different between sexually mature adult and female animals (Deng et al., 2010), we sought to determine if inhibition of estrogen signaling could block the improved recovery of females after ablation. When females were treated overnight with the Esr antagonist Fulvestrant two days post-irradiation, the regenerative advantage of the female fish was

Figure 3.2

A. Exposure to exogenous estrogen enhanced the recovery of hematopoietic progenitors after sublethal irradiation. Additionally, female fish showed greater recovery of progenitors compared to male fish.
B. More proliferating cells, as assessed by PHH3, were found in the kidney marrow of female fish compared to male.

Figure 3.2 (Continued)



impaired (*data not shown*), suggesting that differences in estrogen signaling are one of the key factors that regulate the enhanced regenerative capacity of females compared to males**.** While the mechanisms of sex determination in zebrafish are unknown, and sexual maturation is dependent on nutrient and space resources, zebrafish are well known to exhibit a defined window of fertility, suggesting a peak window of hormone production and a decline in old age (Lawrence, 2007; Liew and Orbán, 2014). To determine if sexual maturity-prepubescence, fertility and aging is correlated with enhanced HSPC regulation and further support the role of estrogen in sex biased hematopoietic phenotypes, we examined irradiation recovery in young zebrafish. Preliminary studies indicate that no difference was found in the in hematopoietic regenerative capacity between the in sexually immature 3-month old fish (*data not shown*). Ongoing studies aim to confirm this finding, as well as characterize hematopoietic recovery in aged fish (>1.5years) that are no longer actively reproducing, to examine sex biases in older animals.

Estrogen Accelerates Hematopoietic Recovery After Irradiation Insult

Given the accelerated HSPC recovery of female fish compared to males after marrow injury and the knowledge that estrogen levels differ between the sexes (Deng et al., 2010), we next examined if exposure of either male or female fish exposed to exogenous E2 could increase the number of hematopoietic progenitors after irradiation. Treatment with E2 overnight two days after irradiation enhanced the number of progenitors in both adult males and females, indicating that E2 can modulate hematopoietic homeostasis in the adult. Treatment with the Esr2 agonist DPN elicited a similar phenotype, implying that it may be the receptor responsible for the observed effects on HSPCs (**Figure 3.2 A**).

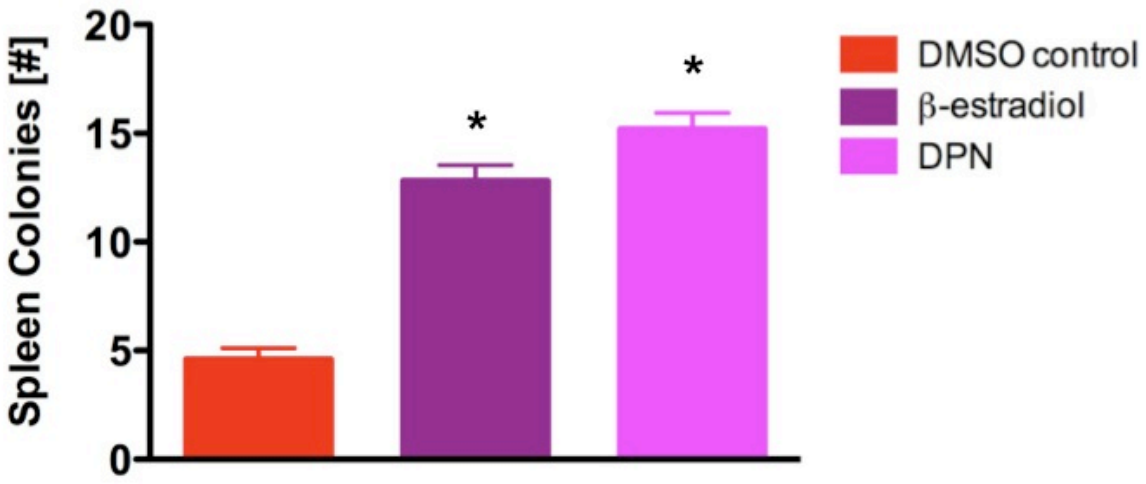


Figure 3.3

Treatment of donor bone marrow with either E2 or the Esr2 agonist DPN enhanced the number of spleen colony units in irradiated recipient mice. (one-tailed t-test *$p<0.05$, error bars indicate standard deviation.)

As estrogen is a known cell cycle regulator (Doisneau-Sixou et al., 2003) and our previous results suggested that one of the means by which E2 increased the number of hematopoietic progenitors during embryogenesis was through increased cell proliferation, the number of cycling cells in the kidney marrow of adult fish was also assessed. Consistent with a role for estrogen in cell cycle control, increased numbers of PHH3+ cells in the kidney marrow were noted in wild-type female fish compared to males (**Figure 3.2 B**), indicating a higher rate of cell division in females. Future work will aim to examine differences in cell cycle regulation after irradiation to assess if E2-mediated differences in this process may account for accelerated HSPC recovery in female fish compared to male.

Estrogen Increases Hematopoietic Progenitor Number in Mice

In order to determine if exposure to exogenous E2 could also increase hematopoietic progenitor number in a murine system, we performed a CFU-S12 assay, which provides a rough measurement of short-term hematopoietic erythro-myeloid progenitor activity (Schofield, 1978). Harvested male bone marrow (BM) cells were pulse treated *ex vivo* with DMSO, E2 or the Esr2 agonist DPN, and a fixed numbers of donor cells were subsequently transplanted into irradiated male recipients (n=10/condition). Twelve days post transplant, spleens were isolated and the number of hematopoietic colonies was counted to assess alterations in the functional progenitor pool mediated by E2 exposure. The number of hematopoietic CFU-S12 progenitors was significantly increased in mice where the BM had been treated *ex vivo* with E2 prior to transplant than in recipients of control BM (**Figure 3.3**). Treatment with the Esr2 agonist DPN also increased hematopoietic progenitor numbers, suggesting physiological boosts of estrogen may have the ability to accelerate hematopoietic recovery in a murine system. Future work will aim to identify if E2 is acting directly on the HSC or if it is mediating its effect indirectly, through modulation of the hematopoietic niche, as well as the long-term functional potential of E2 stimulated donor cells.

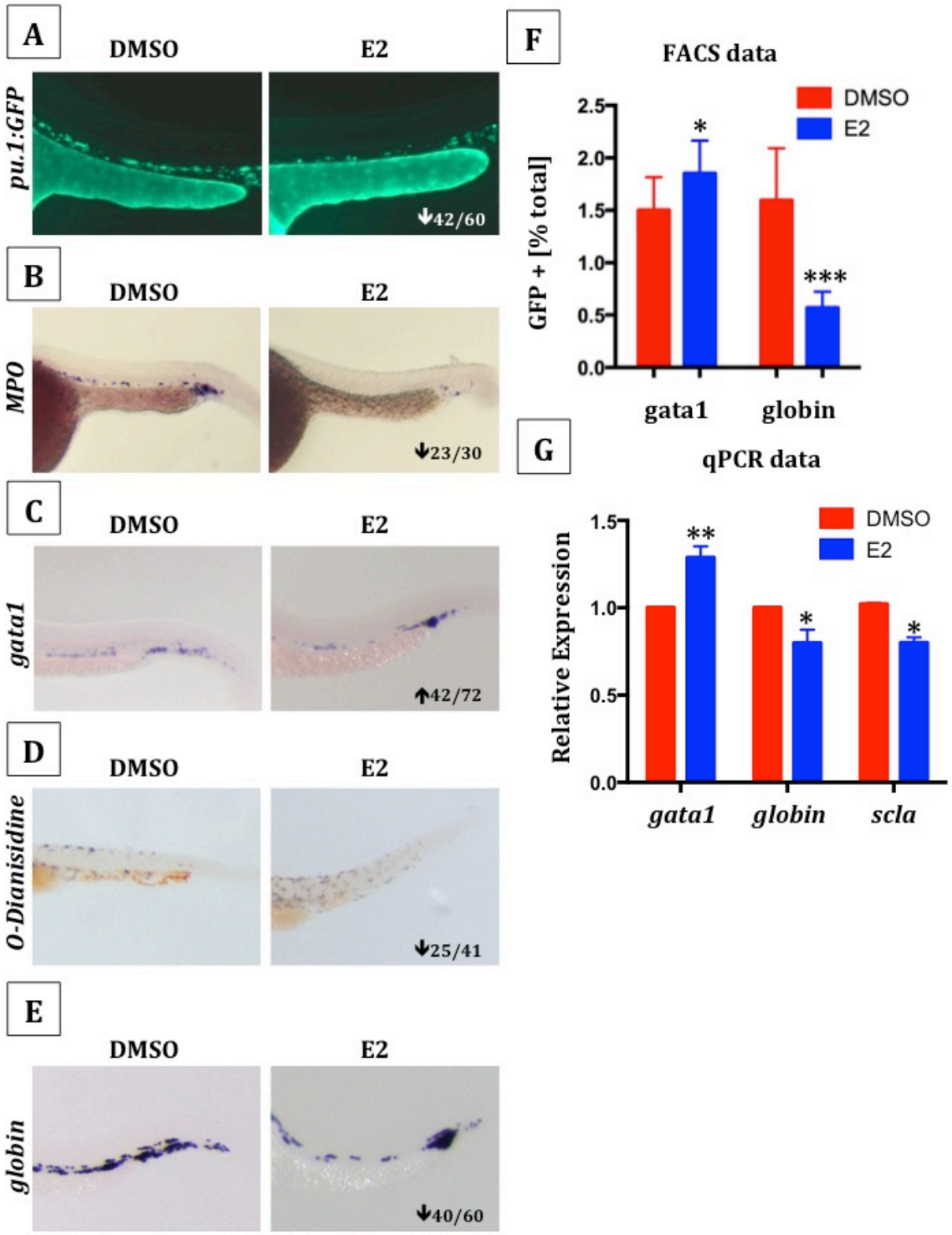
Exposure to Estrogen Alters Primitive Hematopoiesis

As our data suggested that E2 has the ability to regulate HSC homeostasis and function in both the embryo and in the adult, we next sought to determine if it could affect other more differentiated progenitor populations, such as those found during primitive hematopoiesis. When zebrafish embryos were exposed to 10uM E2 from 12-36 hpf,

Figure 3.4

A. Decreased levels of *pu.1* were found after exposure to estrogen (n>50).
B. *MPO* expression was reduced after E2 treatment (n>40).
C. Expression of *gata1* was elevated after estrogen exposure (n>35)
D. Fewer erythrocytes were present following estrogen exposure as assessed by O-Dianisidine (Benzidine) staining (n>50).
E. *globin* expression was diminished after E2 treatment (n>60)
F. FACS analysis of *gata1:GFP* and *globin:GFP* fish confirmed that in situ hybridization observations (one-tailed t-test *p<0.05; error bars indicate standard deviation)
G. qPCR revealed increased levels of *gata1* but lower levels of *globin and scla* after E2 treatment (one-tailed t-test *p<0.05; **p<0.01; error bars indicated SEM).

Figure 3.4 (Continued)



alterations in primitive erythroid and myeloid specification were noted. Consistent with previously published reports indicating a balance between primitive erythropoiesis and myelopoiesis (Galloway et al., 2005), estrogen exposure increased expression of the erythrocyte marker *gata1* by ISH (**Figure 3.4 C**) while simultaneously decreasing the number of *pu.1+* cells in Tg*(spi1b:GAL4,UAS:EGP) (pu.1:GFP)* reporter fish (**Figure 3.4 A**); *MPO* expression was also decreased by *in situ* (**Figure 3.4 B**). The increased expression of *gata1* was confirmed by qPCR (**Figure 3.4 G**) 1-tailed t-test, *p<0.05); using Tg(*gata1:GFP)* transgenic zebrafish, an elevated number of gata1+ cells was enumerated by FACS (**Figure 3.4 F**), indicating increased specification of erythroid progenitors. To determine whether the number of functional erythrocytes was also increased after E2 treatment, O-Dianisidine (benzidine) staining was utilized to assess for the presence of hemoglobin+ (oxygen carrying) erythrocytes. In contrast to results obtained with *gata1,* decreased levels of benzidine stain were noted after estrogen treatment (**Figure 3.4 D**), suggesting that, while E2 treatment leads to increased numbers of erythroid progenitors, their maturation was impaired. To confirm this maturational block, we assessed the expression of *globin*, downstream of *gata1* in erythroid specification. Consistent with the O-Dianisidine results, decreased expression of *globin* was noted by both *in situ* and qPCR (**Figure 3.4 E, G**) 1-tailed t-test, p<0.05). Similarly, fewer globin-positive cells were present by FACS after E2 treatment (1-tailed t-test, p<0.05) in the transgenic Tg*(globin:GFP)* line (**Figure 3.4 F**) Decreased levels of the erythrocyte-specific isoform of *scl,* which is a *gata1* target gene, were also noted by qPCR (**Figure 3.4 G**; 1-tailed t-test, p<0.05). Together, these finding suggest that, while E2 enhanced the number of erythrocyte progenitors, their maturation into functional erythrocytes is impaired.

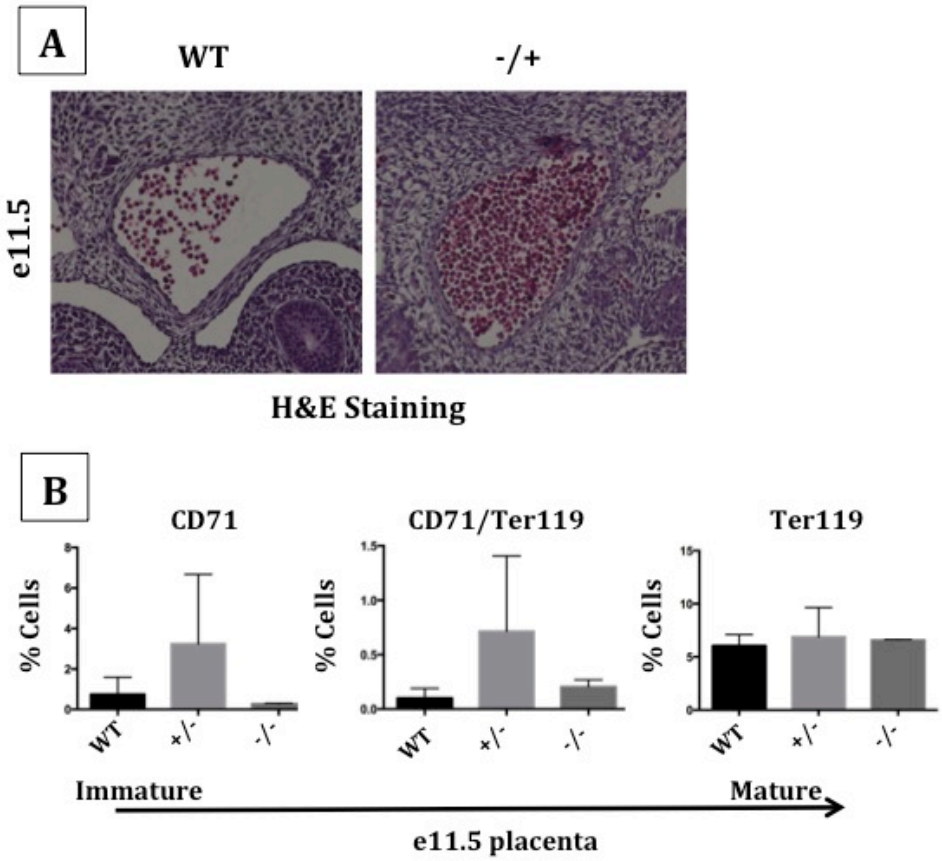


Figure 3.5

A. More erythrocytes were found in the AGM of *srd* mutant embryos compared to WT.
B. Greater number of immature erythrocytes were found in mutant pups compared to wild type. No differences were noted in the number of mature erythrocytes.

E2 Impairs Erythrocyte Maturation in Murine Development

To investigate whether exposure to excess estrogen could similarly block erythrocyte maturation during murine gestation, we utilized 5-α Reductase (*Srd*) mice, which exhibit a 2-3 fold elevation in estrogen levels during pregnancy (Mahendroo et al., 1997). Reminiscent of the results obtained in zebrafish embryos, more erythroid cells were observed in the AGM of mutant animals at E11.5 (**Figure 3.5 A**). To further quantify these changes and assess maturational status, FACS analysis for the erythrocyte cell surface

markers CD71 and Ter119 was utilized (Fraser et al., 2007). Dissociated placental tissue, a site of erythrocyte maturation and expansion in the embryo (Alvarez-Silva et al., 2003; Baron et al., 2012), revealed increased numbers of immature CD71+,Ter119- erythrocytes and CD71+,Ter119+ erythrocytes but no increase in the number of mature CD71+, Ter119+ erythrocytes (**Figure 3.5 B**). Together, these findings suggest erythrocyte maturation is impaired in mice exposed to elevated E2 levels during gestation, just as it is in the zebrafish embryo.

A Potential Interaction between Estrogen and the GATA Factors

While the genetic mechanism underlying the multiple effects of estrogen on HSPCs and erythroid progenitors remains unknown, one intriguing possibility is that an interaction between estrogen receptors and GATA factors may play a role. As detailed above, treatment with estrogen from 12-36 hpf increased expression of *gata1* by qPCR and FACS (**Figure 3.4 C, F-G**); we also noted increased expression of *gata2* in the erythrocytes after E2 treatment in this same window, as well as decreased expression of *gata2* upon antagonism of endogenous estrogen signaling with ZK164015, an estrogen receptor antagonist (**Figure 3.6 B**). Following E2 treatment from 24-36 hpf, after niche specification but correlating with the elevation in HSPC number, increased levels of both *gata2* and *gata3* were noted by qPCR (**Figure 3.6** A; 1-tailed t-test, *p<0.01, ***p<0.001), suggesting that the expression of these genes is modulated at multiple stages of development by estrogen treatment. *Gata2* is known to play a role in both erythrocyte and HSC function (de Pater et al., 2013; Fujiwara et al., 2004; Tsai et al., 1994), making it difficult to know if only one of these populations is responsible for the observed changes in gene expression or if both play a role.

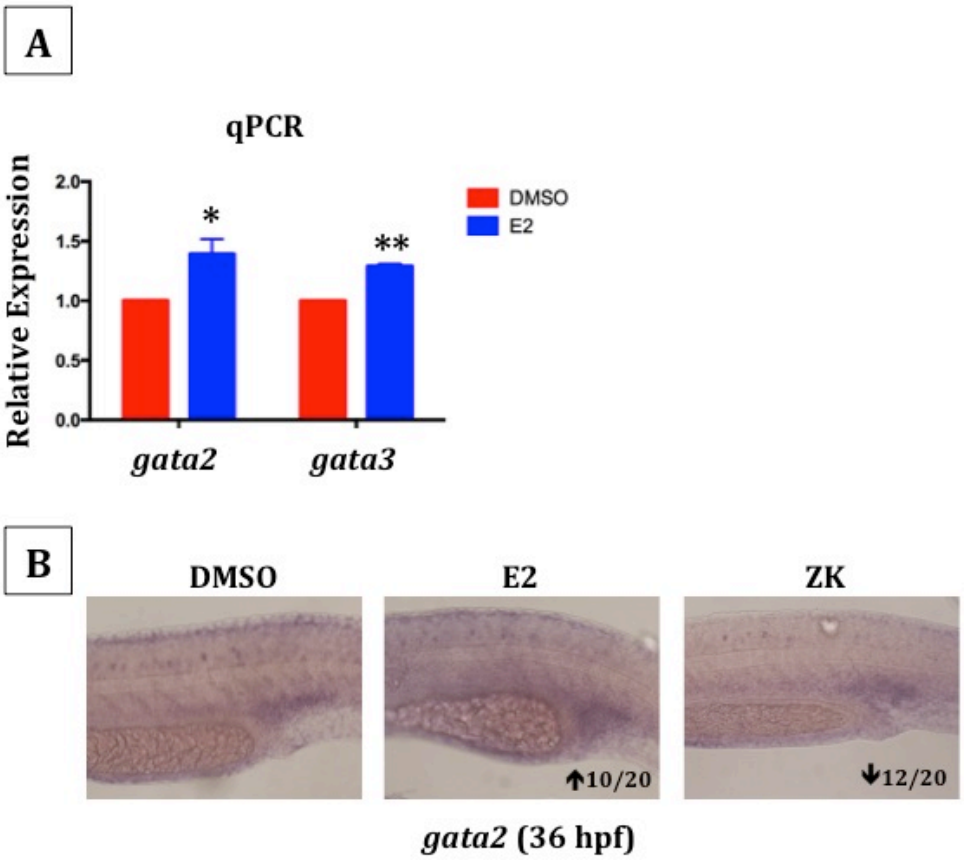


Figure 3.6

A. Expression of both *gata2* and *gata3* was elevated by qPCR after E2 treatment from 26-36 hpf (one-tailed t-test *p<0.05; **p<0.01; error bars indicated SEM).
B. More *gata2* positive erythrocytes were observed after E2 treatment from 12-36 hpf while decreased expression was noted following ZK treatment. (n>20).

Future studies will use cell sorting for either erythrocytes or HSCs followed by qPCR analysis to identify whether there are cell-type specific differences in the expression of the GATA factors after estrogen treatment. Interestingly, there is some evidence that the estrogen receptors and GATA family members can interact. Esr1 has previously been shown to physically bind to GATA1 and impair its function (Blobel and Orkin, 1996; Blobel

et al., 1995). As a result, future work will aim to elucidate a potential interaction between both Esr1 and Esr2 and multiple members of the GATA family to determine if regulation of this set of factors could be an underlying mechanism for many of the hematopoietic phenotypes attributed to E2.

Discussion

Our results indicate that estrogen signaling is a critical regulator of several aspects of hematopoietic homeostasis. We find that HSPCs remain responsive to E2 during development and that estrogen exposure may alter their differentiation capacity and/or lineage potential. As our earlier data indicated a pro-proliferative response, exposure of HSPCs to E2 may bias the cells toward self-renewal or progenitor expansion at the expense of differentiation. In adults, we observed differential regulation of the hematopoietic system between males and females following irradiation and find that the addition of exogenous estrogen can enhance the number of marrow hematopoietic progenitors in both zebrafish and mice after insult by irradiation. Finally, we also confirm that estrogen can modulate other hematopoietic progenitor populations besides the HSC: E2 treatment inhibited primitive myelopoiesis while enhancing the number of erythrocyte progenitors, although their maturation into hemoglobin-carrying erythrocytes was impaired. Together our data implies that estrogen in particular has several regulatory roles in the hematopoietic system and additional studies will be needed to further decipher primary verses secondary effects and niche versus direct HSPC regulatory impact.

While also demonstrated to be under regulatory influence here, erythropoiesis has been suspected of being under hormonal control since at least the 1940s (Vollmer and

Gordon, 1941). Testosterone was used clinically to treat anemia patients as early as the 1960s (Rishpon-Meyerstein et al., 1968; Shahidi and Diamond, 1961) and is still used today (Bachman et al., 2013; Maggio et al., 2013); similarly, anabolic steroids used by professional athletes are testosterone derivatives and are known to stimulate erythropoiesis, one of the reasons for their use (Alén, 1985). Although the majority of studies have focused on the effect of exposure to exogenous testosterone on erythrocyte function, more recent work has indicated that endogenous levels of testosterone also play a role in stimulating erythropoiesis, as low testosterone has been directly associated with a predisposition to anemia in older adults (Ferrucci et al., 2006).

While it remains controversial if the aromatization of testosterone into estrogen is required for the stimulation of erythropoiesis after administration of testosterone (Bachman et al., 2013; Rochira et al., 2009), some evidence suggests that estrogen does have the ability to directly modulate erythrocyte production. However, results have been controversial as to whether estrogen stimulates erythropoiesis (Landshman and Bleiberg, 1979) or if it inhibits it (Mirand and Gordon, 1966). The results obtained here suggest that estrogen may, in fact, both increase the production of erythroid progenitors while simultaneously inhibiting their maturation, helping to reconcile the mixed reports in the literature as to the role of estrogen in erythropoiesis. It will be interesting to determine if co-stimulation with testosterone or timed administration of anastrozole (an aromatase inhibitor) would allow both the boost in erythroid progenitor number and their differentiation toward functional erythrocytes. While the mechanism by which estrogen inhibits erythrocyte maturation remains unknown, future work will aim to more precisely elucidate the block in erythrocyte maturation in order to aid in the identification of the

underlying regulatory action associated with our observations including a closer look at the GATA family of transcription factors already known to play a role in HSC and erythrocytes.

Beyond roles in embryogenesis, our investigation has also indicated that the hematopoietic system is differentially regulated in adult males and females, although the precise mechanisms underlying this difference remain to be established. Recent work from other groups has similarly indicated that the blood system differs between males and females (Nakada et al., 2014), raising profound clinical implications for transfusion and transplantation medicine. Epidemiological studies have indicated that the gender of both the donor and recipient can impact the success of a bone marrow transplant, with the greatest level of success being noted in female-female matches (Gahrton et al., 2005), although this difference was attributed to potential immune system responses to sex-specific factors on the X and Y chromosome rather than differences in hormonal regulation. Donor age has also been reported to influence transplant success, with younger donors having greater engraftment rates than older (Kollman et al., 2001); however, it is not clear how gender may influence the age-related findings. Interestingly, xenotransplantation of human umbilical cord blood into immune compromised mice has been reported to be more efficient in female mice than male, leading to higher sustained chimerism, suggesting sex differences may also impact engraftment potential (McDermott et al., 2010)

One of the most pressing challenges with bone marrow transplantation today is the lack of HLA-matched donors (Copelan, 2006), which has lead to increased use of umbilical cord blood for transplantation when an appropriate donor is not available (Ballen et al., 2013). Interestingly, estrogen levels in cord blood cells are known to be quite variable (Lagiou et al., 2011) and hormone levels have been shown to be associated with cord blood

stem cell potential (Baik et al., 2005). As a result, it would be prudent to assess if differences in E2 levels in donor cells affect the efficacy of cord blood transplantation, as our results suggest this is a possibility. Additionally, transient *ex vivo* treatment of either cord blood or bone marrow with estrogen should be investigated as one means by which to expand the number of donor stem and progenitor cells. The enhancement of spleen colonies in the murine CFU-S12 assay suggests that transient elevations in estrogen signaling could be a highly efficient means of increasing donor cell number, a frequently limiting factor in cord blood transplants (Copelan, 2006). Recent work has suggested that *ex vivo* expansion of donor cord blood cells has the ability to enhance the success of transplantation in adult patients (Cutler et al., 2013; Delaney et al., 2010). However, much work remains to gain a better understanding of the different signals and factors that control stem cell mobilization, migration, and engraftment. Our work suggests that estrogen may be a important factor in this process and indicates that both endogenous estrogen levels as well as donor and recipient sex should be assessed in future studies that examine the efficacy and safety of both cord blood and bone marrow transplants.

Experimental Procedures/Materials and Methods

Zebrafish Husbandry

BIDMC IACUC protocols were followed for all experiments. Zebrafish lines are previously published and described in **Appendix II**. The srd mutant mouse line is previously published (Mahendroo et al., 1997).

Chemical Treatments and Evaluation

Chemical treatments were performed at the following doses: E2 (8-10 μM) and DPN (10 μM), PPT (20 uM). WISH was performed according to established methods (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). Each experiment represents a minimum of three replicates. Phenotypes are scored as the # changed/# examined per treatment. FACS and PHH3 protocols were previously described (Harris et al., 2013; North et al., 2007).

Adult Zebrafish Studies

Adult irradiation experiments were performed as previously described (North et al., 2007). Briefly, adult fish were exposed to a sublethal dose of 20 Gy of irradiation then allowed to recover for 2 days. At 2 days post-irradiation, overnight chemical treatments were performed at the doses indicated above. On day 10, marrows were analyzed by Forward Scatter, Side Scatter as previously described (Traver et al., 2003; 2004).

Murine Assays

CFU-S12 was performed as previously published (North et al., 2007). Murine srd embryos from a heterozygous mating were dissected at E11.5 and prepped for FACS analysis or immunohistochemistry as previously described (Fraser et al., 2007; North et al., 1999). Briefly, embryos were removed from the uterus, microdissected, and disaggregated. Flow cytometry was performed for CD71 and Ter110 (BD Pharmingen); immunohistochemistry for PECAM and ckit (eBioscience).

Chapter 4:

Discussion

The work in this thesis has delineated multiple roles for estrogen signaling during hematopoietic development and regeneration. First, we have identified estrogen as a potent negative regulator of hematopoietic niche specification during early development. We determined that, in zebrafish, maternally derived estrogen acts to define the ventral limit of hemogenic endothelial specification and the localization of HSC emergence within the dorsal aorta through antagonism of somitic-derived VEGF. Second, after the window of niche specification, we observed that estrogen increases the number of embryonic HSCs, in part through enhanced cell cycling. In related studies, estrogen was further noted to elevate the number of primitive erythrocyte progenitors; however, exposure to excess estrogen simultaneously impaired precursor maturation. These results are potentially mediated by interactions with GATA family transcription factors. Finally, we demonstrated that elevated estrogen levels in females are associated with enhanced hematopoietic precursor numbers compared with males after irradiation. Related, we determined that addition of exogenous estrogen has the ability to enhance hematopoietic regeneration after irradiation insult to the marrow in both zebrafish and mice, raising the potential that estrogen status could have important clinical implications or utility.

Estrogenic Regulation of Hemogenic Endothelial Specification

Excess estrogen, during presumed critical intervals, has long been appreciated to have a negative impact on the maintenance of pregnancy, though the mechanisms behind these findings remain poorly understood (Mahendroo et al., 1997; Tong et al., 2005). In the zebrafish, we are able to attribute the reduction in hemogenic endothelial specification after estrogen exposure to the loss of somitic-derived VEGF. In contrast, VEGF activity was

enhanced upon antagonism of the estrogen receptor, indicating that endogenous estrogen found in the yolk normally acts to restrict the ventral-derived limit of VEGF signaling. A recent study in zebrafish indicated that cells fated to become arterial receive higher levels of VEGF signaling relative to those that are venous fated, due to their respective medial and lateral positions in the developing embryo (Kohli et al., 2013). The difference in the fate of these cells was postulated to occur as a result of a gradient of VEGF signaling from the somites. Here, we have identified maternally deposited estrogen as one of the ventrally derived signals that antagonizes this VEGF gradient and helps delineate the ventral limit of arterial identity and subsequent hemogenic endothelial specification. This is a major finding in the field, as the identity of ventral-derived signals acting prior to the sided induction of *BMP4* (Wilkinson et al., 2009), *scl1* (Kim et al., 2013) and *runx1* (Kissa and Herbomel, 2010) in hemogenic endothelium were previously unknown

Future studies will aim to further characterize the conservation of this effect in alternate mammalian models and/or murine strains to assess whether the effects noted in our study are specific to the zebrafish or are more broadly applicable. Recent work has indicated that the signaling cascades regulating the specification of arterial/venous identity and HSC emergence are quite conserved across species (Lindskog et al., 2014), though it remains to be seen if estrogen will also be a cross-species regulator of hemogenic endothelial specification. While not previously documented as it is not anticipated to elicit a lethal phenotype, use of estrogen receptor knockout mice (Krege et al., 1998; Lubahn et al., 1993) should likewise be initiated to determine if a loss of estrogen signaling in a murine system will increase hemogenic endothelium specification and HSC number in a manner similar to that observed in the zebrafish system. Though more study is needed to

determine if excess estrogen signaling in mammalian systems impedes hemogenic endothelium specification, it does appear that carefully regulated estrogen levels are critical for maintenance of pregnancy as both excess and reduced levels have been associated with fetal loss (Albrecht et al., 2000; Mahendroo et al., 1997).

While our work has primarily focused on the impact of excess estrogen in the zebrafish, preliminary studies using 5-α Reductase mice (Mahendroo et al., 1997), which have excess estrogen due to an inability to process testosterone, suggest that at least some aspects of this effect are conserved in a mammalian system, as assessed by decreased expression of CD31 and C-Kit in the AGM of mutant mice. We also noted placental defects and hemorrhaging, consistent with previous work examining excess estrogen content during pregnancy (Tong et al., 2005). During murine gestation, the placenta is thought to be a hematopoietic organ, although it is controversial if the placenta generates HSCs *de novo* or if it acts primarily as a niche to boost the number of HSCs generated in the AGM (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). While our work here does not directly examine if placental defects contribute to the observed hematopoietic failures, future studies should consider this possibility.

Decoupling Hemogenic Endothelial and Arterial Specification

One of the most intriguing findings in the studies on hemogenic endothelium detailed here is the observation that we can induce specification of the HSC marker *runx1* in the absence of arterial identity. We observed this on two separate occasions. First, when *scl* mRNA was injected into a 1-cell embryo and then subsequently treated with estrogen, we noted normal expression of *runx1* by *in situ* hybridization, indicating proper blood

specification, but a complete absence of an *ephrinB2* positive artery. Therefore, we are able to " rescue" the formation of blood without rescuing the loss of arterial identity. Second, and perhaps more importantly, after estrogen antagonism by treatment with ZK164015, we noted the expression of *runx1* in what would normally be venous-specified endothelium, located ventral to arterial tissues. Significantly, in spite of *runx1* expression in the vein, we never noted induction of arterial markers in the area, indicating that, at least in some circumstances, HSCs can indeed be specified in the absence of arterial identity.

Other work has also indicated that in some situations, blood can be induced in non-arterial endothelium. In zebrafish, hyperactivation of the notch cascade resulted in expression of *runx1* in the vein, but no corresponding venous *ephrinB2* induction (Burns, 2005). In the mouse, knockout of the Notch ligand Jagged1 led to a defect in hematopoiesis but normal arterial specification, indicating that arterial identity and specification of HSCs can be decoupled *in vivo* in mammals (Robert-Moreno et al., 2008). Together, these studies raise the question of the necessity of arterial specification for hematopoietic stem cell emergence. Our results corroborate and extend these findings, suggesting that arterial identity alone is not an absolute requirement, nor is it sufficient, for the birth of hematopoietic stem cells. Instead, it is possible that the normally observed overlap in the emergence of hemogenic endothelium and arterial-fated vasculature may be due to their specification by very similar factors including hedgehog, VEGF, and Notch. In our study, we showed using mutant zebrafish lines that the "absolute" concentration of VEGF, and hence downstream Notch activity, was the key factor for specification of *scl+* hemogenic endothelium, implying co-localization of hemogenic endothelium in the artery is more

likely coincident rather than deterministic and suggesting a possibility of identifying downstream or interacting factors that are specific to each separate process.

Estrogen and Erythrocyte Maturation

While early exposure to estrogen was noted to have a potent inhibitory effect on HSC niche specification, treatment with E2 in the same window had a different effect on primitive erythropoiesis. It has been known for decades that males have a higher resting hematocrit than females, which reflects primarily their respective circulating erythrocyte levels (León-Velarde et al., 2000; Valberg et al., 1976; Zeng et al., 2001). Interestingly, while no sex differences were observed in children, hemoglobin and iron levels were found to be lower in females of reproductive age compared to age matched male controls, suggesting hormonal influences over erythrocyte number and function (Valberg et al., 1976). This finding appears to be evolutionarily conserved, as most mammals examined to date show reduced hemoglobin levels in sexually mature females compared to males (Murphy, 2014), though the mechanism behind this finding remains unknown. While most data implicating a role for estrogen in red cell homeostasis has focused on definitive erythropoiesis, our results indicate that when either zebrafish or mice are exposed to excess estrogen during primitive hematopoiesis, increased numbers of primitive erythroblasts are observed but no corresponding increase in mature, oxygen carrying erythrocytes is noted.

One of the most critical transcription factors required for erythrocyte formation and maturation is *Gata1*. When *Gata1* was knocked out of a murine embryonic stem cell line and then injected into a blastocyst that was transferred to a host mother, *Gata1* nulls cells failed to undergo erythrocyte differentiation. Interestingly, the null cells did contribute to

all non-hematopoietic tissues tested as well as white bloods cells, indicating that *Gata1* is particularly critical in erythrocyte function (Pevny et al., 1991). Later studies indicated that *Gata1* null erythrocytes arrest at the proerythroblast stage (Pevny et al., 1995) and that embryos null for *Gata1* die between E10.5 and E11.5 and exhibit anemia (Fujiwara et al., 1996). Interestingly, ESR1 has previously been shown to physically interact with GATA1 and prevent its transcriptional activity (Blobel et al., 1995). Further studies indicated that 24 hours of *in vitro* treatment of Murine Friend virus-inducted erythroleukemia (MEL) cells with E2 induced apoptosis, and that the induction of apoptosis required direct binding of ESR1/GATA1 but not transcriptional activation through estrogen response elements (Blobel and Orkin, 1996). Future work should aim to determine if the physical interaction of Gata1 and the estrogen receptor is indeed resulting in a failure of erythrocyte maturation after estrogen exposure during primitive hematopoiesis in both the zebrafish and the mouse.

Interestingly, in zebrafish, *gata1* null animals have different phenotypes depending on the severity of the mutant allele utilized in each study. Vlad tepes fish, which possess a null mutation in *gata1*, show a complete loss of erythropoiesis and are embryonic lethal (Lyons et al., 2002). However, in zebrafish with an ENU-induced *gata1* hypomorphic mutation, the number of primitive erythrocytes was reduced, but not absent while definitive hematopoiesis was unaffected (Belele et al., 2009). These findings suggest that the level of *gata1* is critical in determining hematopoietic function and that relatively higher levels of *gata1* are required for primitive hematopoiesis compared to definitive. As a result, the reduction in primitive erythropoiesis after estrogen exposure we observed here is likely due to a reduction in *gata1* activity rather than a complete loss of function, as we

do note some mature erythrocytes. Recent work has suggested that an interaction between GATA1 and GATA2, through a process termed GATA switching, is critical for erythrocyte maturation. During GATA switching, GATA1 is thought to displace GATA2 at GATA chromatin sites, thereby leading to differential transcriptional activation or repression (Bresnick et al., 2012; 2010). As we observed induction of both *gata1* and *gata2* by E2, it will be interesting to assess if differences in GATA switching at key erythrocyte genes may play a role in the observed defects in erythrocyte maturation. If, as has been suggested, E2 impairs the function of GATA1 by direct binding (Blobel and Orkin, 1996; Blobel et al., 1995), it is possible that GATA1 may be unable to displace GATA2 at important regulatory genes, thereby resulting in a block in erythrocyte maturation and a sustained progenitor phenotype.

Enhancing Hematopoietic Regeneration via Enhancement of Estrogen Signaling

When zebrafish were exposed to E2 after the specification of hemogenic endothelium, a robust increase in the expression of HSC markers was initially noted. However, during primitive hematopoiesis, while we likewise observed increased numbers of erythrocyte precursors, their function was impaired. Similarly, further characterization during definitive hematopoiesis indicated that although we observed more hematopoietic progenitors, we noted a reduction in the specification of the myeloid and lymphoid lineages. This finding suggests that the maturation and functional capacity of the expanded population of HSCs may be impaired after exposure to excess E2. While the mechanism behind this observation remains unknown, one intriguing possibility is that the GATA factors are playing a key role in this process as well. We observed induction of both *gata2*

and *gata3* following estrogen treatment after niche specification. A GATA2/3 switch has been proposed to play a role in placental development (Ray et al., 2009), and may be important in other developmental processes (Bresnick et al., 2010; 2012). It will be interesting to explore if the estrogen receptors (either Esr1 or Esr2) have the ability to bind to other GATA factors besides Gata1, which has been previously reported (Blobel and Orkin, 1996; Blobel et al., 1995) and if switches in the occupancy of various chromatin sites by Gata2 and Gata3 differ after E2 exposure in the definitive wave, as both *Gata2* and *Gata3* are important in HSC development (de Pater et al., 2013; Fitch et al., 2012).

In addition to regulation of HSPCs by estrogen during development, the ability of E2 to increase the number of HSPCs was conserved in the adult where exposure to a short burst of E2 accelerated hematopoietic recovery after irradiation. Consistent with a positive effect of E2 in HSC recovery after irradiation, female fish recovered better from irradiation injury to the marrow than did male fish, indicating an inherent sex bias in the regenerative capacity of the zebrafish hematopoietic system. While more work is required to demonstrate that the enhanced regeneration of the female fish is indeed due to their elevated estrogen levels and that the increase in precursor content translates to enhanced numbers of effector cells (or a differentiation block), it is intriguing that the regenerative capacity of the fish appears to be sex-dependent.

One of the uncertainties raised by these findings is whether the ability of estrogen to accelerate hematopoietic recovery is due to the effect of E2 on the niche or due to a direct effect on the HSC. In the mouse, estrogen is a well-known modulator of bone homeostasis (Weitzmann and Pacifici, 2006). However, since the bone is a critical component of the hematopoietic niche (Morrison and Scadden, 2014), it is difficult to assess if the effects of

E2 on hematopoiesis are niche-dependent or independent. As the zebrafish kidney marrow is bone-independent, it may be a better model in which to ask if the effects of E2 on HSC regeneration is direct or indirect. Future studies will aim to answer this question via the use of zebrafish transplantation experiments where the sex of the donor and recipient are varied. It will also be important to determine if the different lineages of the blood are properly specified after hematopoietic regeneration or if a lineage bias or differentiation block is noted after recovery by allowing more long-term regeneration. These experiments will be crucial for assessing the potential utility of E2 in a clinical context to improve engraftment of bone marrow or cord blood cells during transplantation.

Our results in the murine CFU-S12 assay indicate that the ability of estrogen to increase HSPC number is conserved in a mammalian system. This finding is consistent with recently published data suggesting that E2 increases the number of HSCs in the marrow through enhanced cell cycling (Nakada et al., 2014). It has also been reported that female immune compromised mice show enhanced engraftment of transplanted human cord blood cells compared to male mice (McDermott et al., 2010), suggesting that the microenvironment or niche of the recipient may play a critical role in the observed engraftment differences.

Another intriguing possibility that arises due to our observation of an enhanced hematopoietic regenerative capacity in female zebrafish compared to males is whether this is a blood-specific phenomenon or if it will be true in other organ systems as well. Several pieces of evidence suggest that estrogen, or hormones in general, may be broadly pro-regenerative. Zebrafish are well-known to regenerate their tail fins, and a recent study indicated that male fish had impaired tail regeneration compared to female fish due to

differences in localized androgen signaling (Nachtrab et al., 2011) as well as inhibition of the Wnt pathway (Kang et al., 2013). Estrogen itself has been suggested to enhance regeneration of both the liver (Kitagawa et al., 2009) and sciatic nerve (Islamov et al., 2002), suggesting that the blood system is not the only organ that is estrogen sensitive. In the highly regenerative MRL mouse strain, female mice show enhanced regenerative capacity compared to males (Blankenhorn et al., 2003); likewise, female-derived muscle cells were shown to be more efficient at regeneration than male cells (Deasy et al., 2007). Though neither of these studies indicated that estrogen was responsible for the observed sex differences, these findings still suggest that there may be interesting regenerative sex biases that warrant further study. It will also be intriguing to determine if similar differences are observed in humans or if they are specific to lower vertebrates.

Estrogen and Pregnancy-Related Changes in Blood

While the concept of estrogen as a pro-regenerative compound is not well elucidated, estrogen has long been suspected of being a regulator of hematopoietic homeostasis due to the critical role that elevated estrogen levels play in the maintenance of pregnancy. Maternal blood volume increases during gestation in order to meet the enhanced nutritional and oxygenation needs of the fetus during development (Hytten, 1985); related, artificially raising estrogen levels has been shown to induce changes in blood volume that are very similar to those noted during gestation, indicating that estrogen is one of the critical modulators of this process (Davis et al., 1989).

Given our observations suggesting that excess estrogen has a detrimental effect during certain windows of development, together with data suggesting that estrogen is

elevated during pregnancy (Tulchinsky et al., 1972), it raises the question of how the embryo is normally protected from the high estrogen levels found during pregnancy. Several mechanisms appear to be in place. First, enzymes that degrade estrogen entering into the fetus from the placenta are found at high levels in the umbilical arteries, and are proposed to be one means of decreasing fetal estrogen exposure from maternal circulation (Simard et al., 2011). Further, it is likely that estrogen is normally degraded and/or inactivated *in vivo* as failure to degrade E2 leads to fetal loss and placental defects during gestation, underscoring the importance of this process for embryonic viability (Tong et al., 2005). Similarly, careful regulation of testosterone levels is critical for controlling estrogen levels during pregnancy, as a failure to metabolize testosterone during murine gestation led to increased E2 levels and fetal loss (Mahendroo et al., 1997). As a result, it is likely that, while maternal E2 levels are quite high during gestation (Tulchinsky et al., 1972), various mechanisms are in place to prevent embryonic exposure to excess estrogen.

One of the most intriguing findings of this thesis is that estrogen has the ability to regulate hematopoietic homeostasis both outside the context of pregnancy and in an animal that does not undergo placental or *in utero* gestation. This observation suggests that the ability of estrogen to modulate hematopoiesis may have evolved prior to the development of *in utero* gestation. Consistent with this fact, based on the sequence homology between vertebrate ERs and invertebrate steroid receptors, the first steroid hormone receptor is believed to have been an estrogen receptor (Eick and Thornton, 2011) and to have evolved at least 450 million years ago (Thornton, 2001). Animals as primitive as annelids (Keay and Thornton, 2009) and mollusks (Thornton et al., 2003) have been shown to possess an estrogen receptor, indicating its ancient role in evolutionary history.

These findings suggest that estrogen is a critical and highly conserved hormone and that it may play long-standing roles in hematopoietic homeostasis outside of its more prominent role in sexual reproduction and maturity. Similarly, hematopoiesis is more evolutionarily ancient than hematopoietic stem cell formation (Hartenstein, 2006). Therefore, it is possible that estrogen may have had played an early role in hematopoiesis that was then co-opted as a means to expand hematopoietic output as organisms evolved and became more specialized in their function.

The observation that estrogen has the ability to influence hematopoiesis in an organism that does not undergo *in utero* or placental pregnancy suggests that alterations in estrogen signaling are a crucial and evolutionary ancient means by which to control hematopoietic homeostasis. It also raises the intriguing possibility that, rather than estrogen signaling evolving to support pregnancy and the associated increases in blood volume, pregnancy may have "hijacked" the already established ability of estrogen to regulate hematopoiesis to ensure that blood volume increases to a sufficient level to meet the needs of the mother and fetus during gestation. Though further work will be needed to confirm this hypothesis, our findings here suggest that estrogen signaling will have the ability to modulate hematopoietic homeostasis in many diverse species and outside the context of pregnancy and sex-related hematopoietic differences.

Conclusion and Larger Implications

While great strides have been made in enhancing our knowledge of both the development and regeneration of the hematopoietic system, much remains unknown. The work detailed in this thesis has deepened our knowledge of hematopoiesis in several

different ways. We have identified estrogen signaling as having multiple effects on hematopoietic development, including a key role in the specification of hemogenic endothelium and, after niche development, the ability to enhance the number of HSPCs in the embryo. We also identify estrogen as potently impeding the maturation of erythrocytes in both the zebrafish and the mouse. Finally, we demonstrate that exposure to exogenous estrogen after hematopoietic ablation by irradiation enhances the recovery of hematopoietic progenitors while also identifying an innate sex bias in the course of hematopoietic stem and progenitor regeneration.

Together, these findings enhance our knowledge of multiple aspects of hematopoietic development including the identification of signals that specify the regional localization of HSC emergence. Future work will aim to examine these processes in more detail as well as identify conservation of effect in murine or human HSC biology and the mechanisms by which they occur. The inherent sex biases we observed also raise interesting clinical questions that remain to be answered. For instance, our work indicates that HSCs derived from female patients may be more potent than those derived from males and could suggest that donor sex (as well as age) need to be taken into account in identification of potential donors for patients undergoing transplantation. Similarly, if the regulation of the hematopoietic system is different in males and females as both our work and the work of others (Nakada et al., 2014) indicates, it suggests that scientists and clinicians should consider gender when studying both hematopoietic homeostasis as well as hematopoietic malignancies and that combining results across sexes could minimally complicate experimental analyses.

As our knowledge of hematopoietic stem cell biology grows, these questions will gain increasing importance as we seek to understand the regulation of the blood system and ways that this knowledge can be exploited for clinical use. Estrogen signaling appears to be a key regulator of multiple aspects of hematopoiesis and will remain an important molecule for future study. Advances in our understanding of sex differences in homeostasis, regeneration, and disease have profound implications for the care and treatment of patients who suffer from hematological disorders. Future work must take these differences into consideration to bolster our understanding of the hematopoietic system and the signals necessary for its control.

Appendix I:

Cannabinoid Receptor-2 Signaling Regulates Embryonic Hematopoietic Stem Cell Function via Prostaglandin E2 and P-selectin

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Attributions

Cannabinoid signaling was identified as a modifier of *runx1/cmyb* expression by WG and TEN. VE performed initial experiments to identify the time-dependent effects of CNR2 stimulation on HSC production and function as well as identification of the PGE2 and p-selectin pathways, respectively, as downstream mediators of these effects. KJC performed mechanistic experiments involving CNR2 and the PI3K and ERK pathways as well as qPCR quantification, and MO injections for p-selectin signaling analysis. VE, KJC, WK, MC, and SL performed biochemical assays and FACS analysis. VE and MC performed Western Blots. GMF, SL and LV did embryo exposures, qPCR, and in situ hybridizations. VE, KJC, WG, and TEN designed experiments and evaluated results. VE and TEN wrote the manuscript.

Introduction

In vertebrates, definitive Hematopoietic Stem Cells (HSCs) give rise to all the mature blood cell lineages of an individual throughout its lifetime. Embryonic and adult HSCs interact with specialized microenvironments or niches, which are required for their function (Cao et al., 2013). In normal and pathologic conditions, HSCs dynamically respond to environmental cues to expand, differentiate, or inhabit the bone marrow (BM) niche. The processes of HSC mobilization and homing are tightly regulated by chemo-attractants and adhesion molecules that are co-opted in therapeutic applications such as HSC transplantation (HSCT), the oldest successful stem cell-based therapy (Thomas et al., 1957); Understanding how HSCs move in and out of their regulatory niche is critical to improve efficiency of HSC mobilization and homing in the clinical setting.

The transcription factor *Runx1* is required for HSC development (North et al., 1999; 2002), where it functions in endothelial-to-hematopoietic transition (Chen et al., 2009). Hematopoiesis is well conserved among vertebrates (Orkin and Zon, 2008); in zebrafish, the first HSPCs, marked by *runx1,* emerge in the aorta-gonad mesonephros (AGM) region around 27 hours post fertilization (hpf) (Bertrand et al., 2008); these newly produced HSPCs upregulate expression of other conserved markers: *cmyb* and *cd41* (Bertrand et al., 2008; Kissa et al., 2008). Starting at 32-34hpf, HSPCs migrate to the caudal hematopoietic tissue (CHT), an intermediate site of hematopoiesis, to expand and mature while mature HSCs seed kidney marrow (KM) and thymus beginning at 48-60hpf (Bertrand et al., 2008; Kissa et al., 2008). Though the embryonic migration routes of HSPCs are well characterized in zebrafish (Bertrand et al., 2008; Kissa et al., 2008; Murayama et al., 2006), the molecular

mechanisms underlying the migration process, including the rationale for successive niches, remain elusive.

In murine embryos, HSCs exhibit a maturation-dependent expression profile of adhesion molecules and chemokines thought to reflect their ability to colonize subsequent hematopoietic organs (Ciriza et al., 2012). Adhesion molecules including Cadherins, Integrins and E-/P-Selectins have been associated with migration of HSCs to the fetal liver (FL) and BM (Ciriza et al., 2012). In the adult, these molecules regulate migration through the endothelium and extracellular matrix (ECM), as well as anchoring, to control HSC homing and BM retention (Sahin and Buitenhuis, 2012). Chemokines, cytokines and their receptors regulate fetal HSC movements by establishing gradients for directional migration (Ciriza et al., 2013; Sahin and Buitenhuis, 2012); CXCR4/CXCL12 (SDF-1) are crucial for HSC transit from the FL to the BM and thymus (Bleul and Boehm, 2000; Calderón and Boehm, 2011; Ciriza and García-Ojeda, 2010; Ciriza et al., 2012), and BM retention. Finally, proteases, such as the Matrix Metalloproteinases (MMPs), affect HSCs through ECM degradation and proteolytic activation of chemokines and growth factors (Shirvaikar et al., 2012). While not conclusively demonstrated, seeding of the shifting hematopoietic niches during ontogeny is thought to enable proper maturation of the HSC pool through differential microenvironmental signals (Cao et al., 2013); however, it is unclear how these factors are integrated to influence the production and expansion of HSCs during embryogenesis, or transit to and from sequential developmental niches.

Concomitant with HSC induction and migration during development, there is a rapid expansion in HSC number: between E12.5-E14.5 murine FL HSCs double as a result of cell proliferation (Morrison et al., 1995). In the zebrafish CHT, the HSC pool similarly expands (Murayama et al., 2006), although the precise rate of proliferation has not been established (Alghisi et al., 2013; Hirabayashi et al., 2013; Li et al., 2012). A variety of factors can increase HSC proliferation *in vivo* and *in vitro* in adult HSCs (Walasek et al., 2012). In contrast, relatively little is known about the regulation of HSC expansion during development. However, roles for HIF1α, IGFs, Wnt and PGE2 have been elucidated (Austin et al., 1997; Goessling et al., 2009; Harris et al., 2013; Luis et al., 2009; North et al., 2007). Interestingly, several factors involved in BM HSC retention and migration, including Selectins and CXCR4/CXCL12, also appear to influence cell proliferation (Eto et al., 2005; Lévesque et al., 1999; Mo et al., 2013; Winkler et al., 2012), suggesting they may contribute to multiple uncharacterized facets of embryonic HSPC regulation.

Eicosanoids are a family of lipid modifiers related to arachidonic acid (AA) that includes epoxyeicosatrienoic acids, leukotrienes, prostaglandins and endocannabinoids (ECs). Many are known modifiers of adult HSC function (Hoggatt and Pelus, 2010), and their synthesis/signaling pathways are highly interconnected. Prostaglandin E2 (PGE2) is the best characterized in relation to HSC regulation. During zebrafish development, PGE2 modulates Wnt-signaling to control HSC production, while in adult fish or mice, PGE2 enhances hematopoietic regeneration after injury by increasing HSC proliferation and survival (Goessling et al., 2009; North et al., 2007). In transplantation assays, PGE2 increases BM homing in a CXCR4-dependent manner (Goessling et al., 2011; Hoggatt et al.,

2009). ECs 2-arachidonoylglycerol (2-AG) and anandamide (AEA) similarly modulate cell proliferation and migration of hematopoietic cells *in vitro* (Jordà et al., 2002; Joseph et al., 2004; Kishimoto et al., 2005; 2006; Oka et al., 2004; Valk and Delwel, 1998; Valk et al., 1997). Endogenous and synthetic CBs bind to G-protein coupled receptors: CNR1, CNR2 and GPR55, with differing affinities (Pertwee et al., 2010); while CNR2 is predominantly expressed on immune cells (Galiègue et al., 1995), both CNR1 and CNR2 are expressed on immuno-phenotypically defined HSCs (Hoggatt and Pelus, 2010). *In vivo*, CNR2-signaling increases recovery after irradiation through modulation of HSPC proliferation and apoptosis (Jiang et al., 2011); administration of the non-selective CNR-agonist CP55940, or CNR2-agonists, was sufficient to mobilize CFU-GM into the peripheral blood and enhanced the efficiency of G-CSF-mediated mobilization (Hoggatt and Pelus, 2010). Together, these data suggest CNR2 can induce both proliferative and migratory behavior of adult HSPCs. However, the impact of CBs on embryonic HSC formation, expansion and/or seeding of adult hematopoietic organs remains unknown.

Here, we demonstrate a role for cannabinoids during hematopoiesis in zebrafish. Exposure of embryos to ECs or CNR2-selective agonists increases the number of *runx1*$^{+}$ HSPCs. Using discrete windows of exposure to CNR2-modulating compounds, we show CNR2 stimulation by AM1241 during niche specification (18-24hpf) enhances AGM HSC development, while treatment during the time of HSC production (30-36hpf) and colonization (4dpf) alters the number of HSPCs appearing in subsequent niches: CHT and thymus. Blockade of CNR2-signaling in either time window decreased the total number of HSPCs in the embryo. Using genetic and chemical approaches, the effect of AM1241 in the AGM was determined to be

mediated by up-regulation of PGE2 synthesis and signaling. In contrast, the impact of CNR2 stimulation on HSPCs in the CHT and thymus is PGE2-independent, requiring the P-selectin pathway. Together, these data uncover a novel role for CBs as modulators of HSC specification and migration during vertebrate embryogenesis.

Results

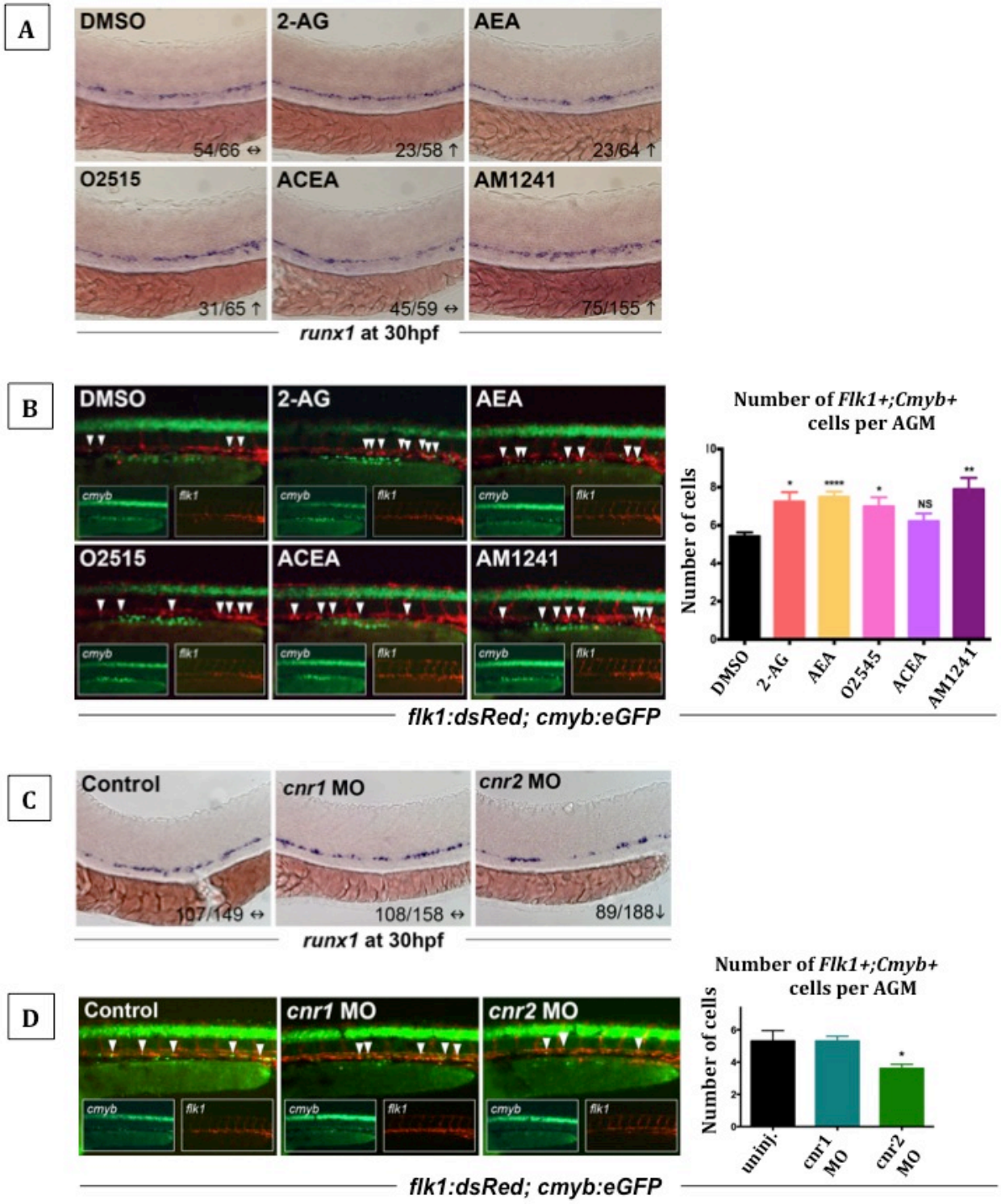
CNR2-signaling increases AGM HSPCs and is required for their normal development.

PGE2 is a known modulator of HSC development and homeostasis in vertebrates (North et al., 2007). To determine if related eicosanoids could similarly impact embryonic HSC production and function, we assessed the effect of endogenous and synthetic CB. Exposure of zebrafish embryos to 2-AG and AEA during hematopoiesis (12-30hpf), increased AGM *runx1* expression by *in situ* hybridization; O2515, a dual CNR1/CNR2-agonist, elicited a similar response (**Figure 4.1 A**). Whereas embryos exposed to the CNR1-agonist ACEA exhibited no effect on *runx1*, CNR2-agonists AM1241 and JWH015 each recapitulated the EC phenotype. These results where confirmed by fluorescence microscopy of *flk1:dsRed*; *cmyb:GFP* embryos (**Figure 4.1 B**); quantification by absolute cell counts of dual positive *flk1:dsRed*;*cmyb:GFP* HSPCs in the AGM revealed a significant enhancement following EC exposure which appeared to be due to CNR2 stimulation. To confirm receptor-specificity and examine the requirement for EC function, *cnr1* and *cnr2* were knocked down using modified antisense oligonucleotides (morpholino, MO). While the *cnr1* MO had no effect, *cnr2* morphants exhibited a reduction in *runx1* expression at 30hpf (**Figure 4.1 C**); this was confirmed and quantified by fluorescence microscopy of *flk1:dsRed*;*cmyb:GFP* embryos at 36hpf (**Figure 4.1 D**), showing a significant reduction in HSPC production with loss of *cnr2*.

Figure 4.1

A. Exposure of WT embryos to various endogenous (2-AG; AEA) and synthetic (O2515, CNR dual-selective; ACEA, CNR1-selective; AM1241, CNR2-selective) cannabinoid compounds during the time of HSC development (12-30hpf) revealed that CNR2 stimulation increased the number of *runx1*$^{+}$ HSPCs in the AGM (DMSO: 54/66=, 2-AG: 23/58↑, AEA: 23/64↑, O2515: 31/65↑, ACEA: 45/59=, AM1241: 75/155↑).
B. Counts of double positive *flk1:dsRed; cmyb:egfp* embryos confirmed the increase in *runx1* expression by *in situ*. (*p<0.05, **p<0.01, ****p<0.001).
C. Knock-down of *cnr1* or *cnr2* by morpholino (MO) injection showed that CNR2, but not CNR1, was required for normal *runx1*$^{+}$ HSPC development (Uninjected: 107/149=, *cnr1* MO: 108/158=; *cnr2* MO: 89/188↓).
D. *In vivo* imaging of *flk1:dsRed*; *cmyb:egfp* embryos showed that the number of *flk1*$^{+}$*;cmyb*$^{+}$ HSCs (arrowheads) were decreased in the AGM at 36hpf following *cnr2* but not *cnr1* inhibition (Uninjected: 5.3±0.7, *cnr1* MO: 5.3±0.3, *cnr2* MO: 3.6±0.3, p<0.027).

Figure 4.1 (Continued)



In situ hybridization for *cnr2* revealed low levels of expression throughout the embryo, including within the AGM region at 36 hpf and 48 hpf (**Figure 4.2 A**). qPCR analysis of FACS-sorted 48 hpf embryonic fractions confirmed *cnr2* was present in *flk1:dsRed+;cmyb:GFP+* hemogenic endothelium and/or newly produced HSPCs (**Figure 4.2 B**). Together, these data show CNR2-signaling positively modulates HSPC production and is active during definitive hematopoiesis in the embryo.

CNR2 stimulation can modulate HSPC development in distinct hematopoietic tissues.

The development and function of embryonic HSPCs can be divided into four broad, partially overlapping phases: (1) hematopoietic niche specification (18-24hpf); (2) AGM HSPC production (30-48hpf); (3) CHT colonization and HSPC expansion (from 34 to 72hpf) (Jin et al., 2007; Kissa et al., 2008; Murayama et al., 2006); (4) kidney marrow (KM) and thymus colonization (>2-5dpf) (Bertrand et al., 2008; Kissa et al., 2008) (**Figure 4.3 A**). To determine the processes affected by CNR2-signaling, embryos were exposed to AM1241 during discrete intervals and HSPCs assessed in each hematopoietic site. Following AM1241 treatment during niche specification (18-24hpf), *runx1/cmyb* expression in the AGM increased at 30hpf; a decrease in the number of HSCPs was confirmed by fluorescence microscopy of *runx1P1:egfp* embryos (**Figure 4.3 B**) and quantified by *cmyb:eGFP*$^{+}$*;lmo2:dsRed*$^{+}$ FACS analysis (**Figure 4.3 B**). In contrast, exposure to the CNR2-selective antagonist AM630 during niche specification significantly decreased, but did not eliminate, expression of *runx1*$^{+}$ (**Figure 4.3 C**). Absolute counts of *flk1:dsRed*$^{+}$;*cmyb:eGFP*$^{+}$ cells in the AGM of confirmed HSC modulation by both cnr2 stimulation and suppression

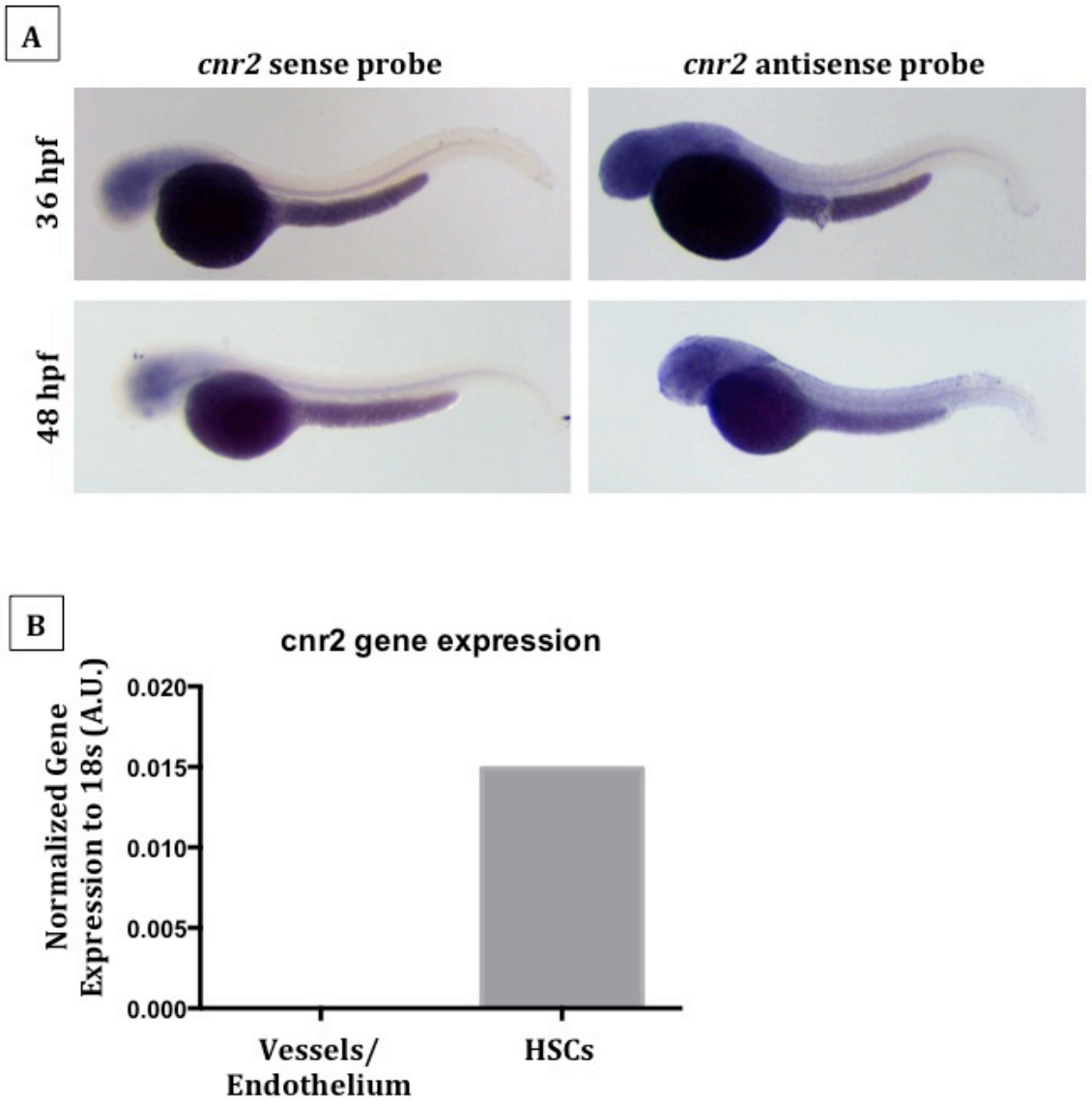


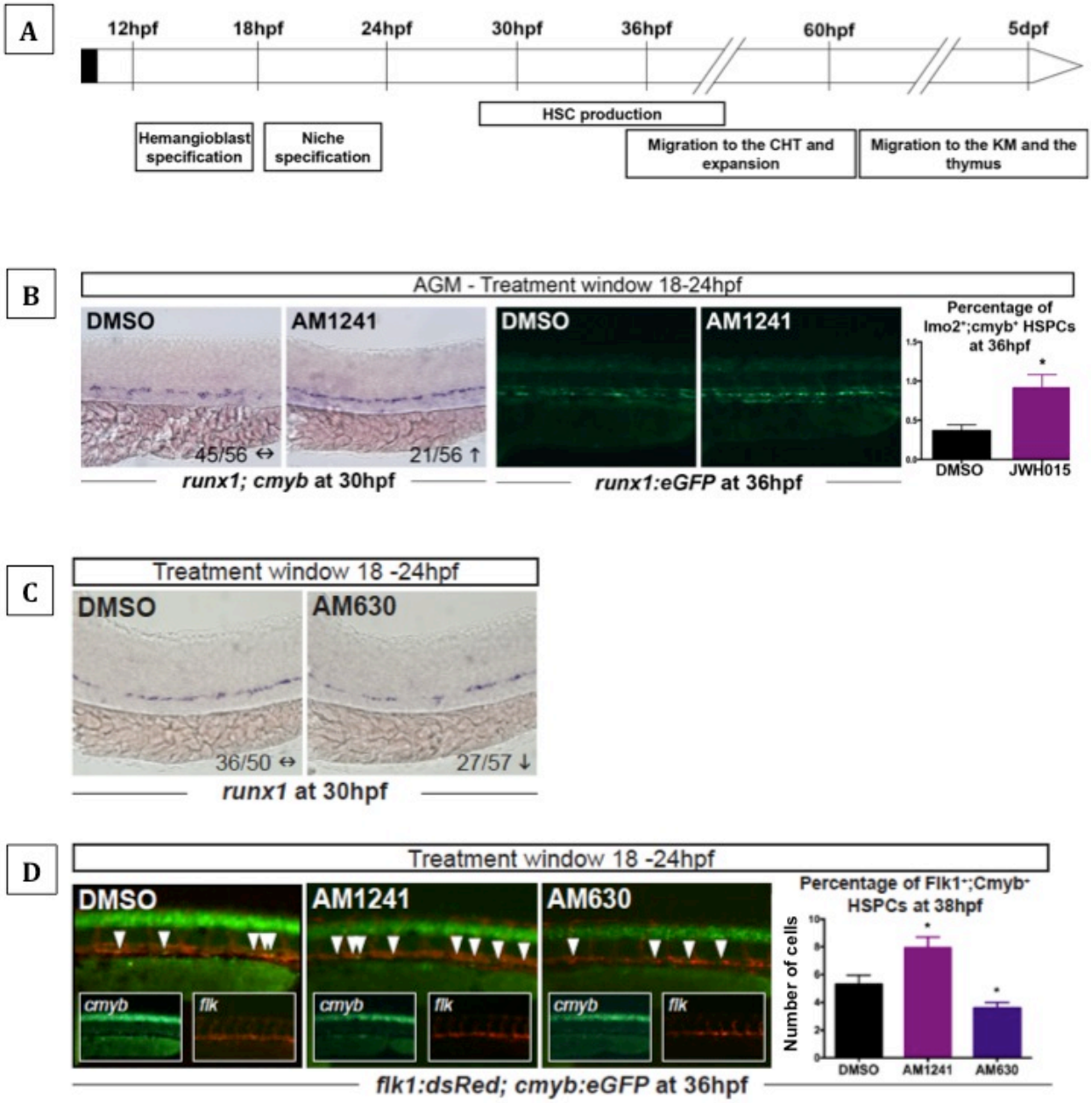
Figure 4.2

A. *cnr2* was broadly expressed in the embryo at both 36 and 48 hpf.
B. *cnr2* was preferentially expressed in *flk:dsRed/cmyb:GFP* dual positive HSCs but was not expressed in the vasculature (*flk.dsRed+/cmyb:GFP-*).

Figure 4.3

A. Scheme representing the different stages of HSPC development starting during somitogenesis (12hpf) and continuing until larval stages (5dpf).
B. Embryos exposed to AM1241 during niche specification (18-24hpf) exhibited elevated expression of *runx1*;*cmyb*$^{+}$ in the AGM at 30hpf (DMSO: 45/56=, AM1241: 21/56↑). *In vivo* imaging of *runx1:egfp* embryos confirmed that the number of Runx1^{+} HSPCs was increased in the AGM at 36hpf following AM1241 exposure. The effect of CNR2 stimulation, using JWH015, was quantified by FACS using *cmyb:gfp*;*lmo2:dsRed2* dual transgenic line (2.48-fold, $p<0.018$).
C. Embryos exposed to the CNR2 antagonist AM630 during niche specification (18-24hpf)exhibited a decreased number of *runx1*-expressing HSPCs in the AGM (DMSO: 36/50=, AM630:27/57↓).
D. During niche specification, in vivo imaging of *flk1:dsRed;cmyb:egfp* embryos showed that the number *Flk1+;Cmyb*+ HSCs were increased or decreased in the AGM at 36hpf following CNR2 agonist (AM1241) and antagonist (AM630) exposure, respectively (DMSO: 5.3±0.7 *flk1+;cmyb*+ cells per AGM, AM1241: 7.9±0.8, $p<0.021$, AM630: 3.6±0.4, $p<0.039$, $n>10$).

Figure 4.3 (Continued)



(**Figure 4.3 D**). Together, these results show that CNR2-signaling functions during niche specification (18-24hpf) to modulate HSPC production in the AGM.

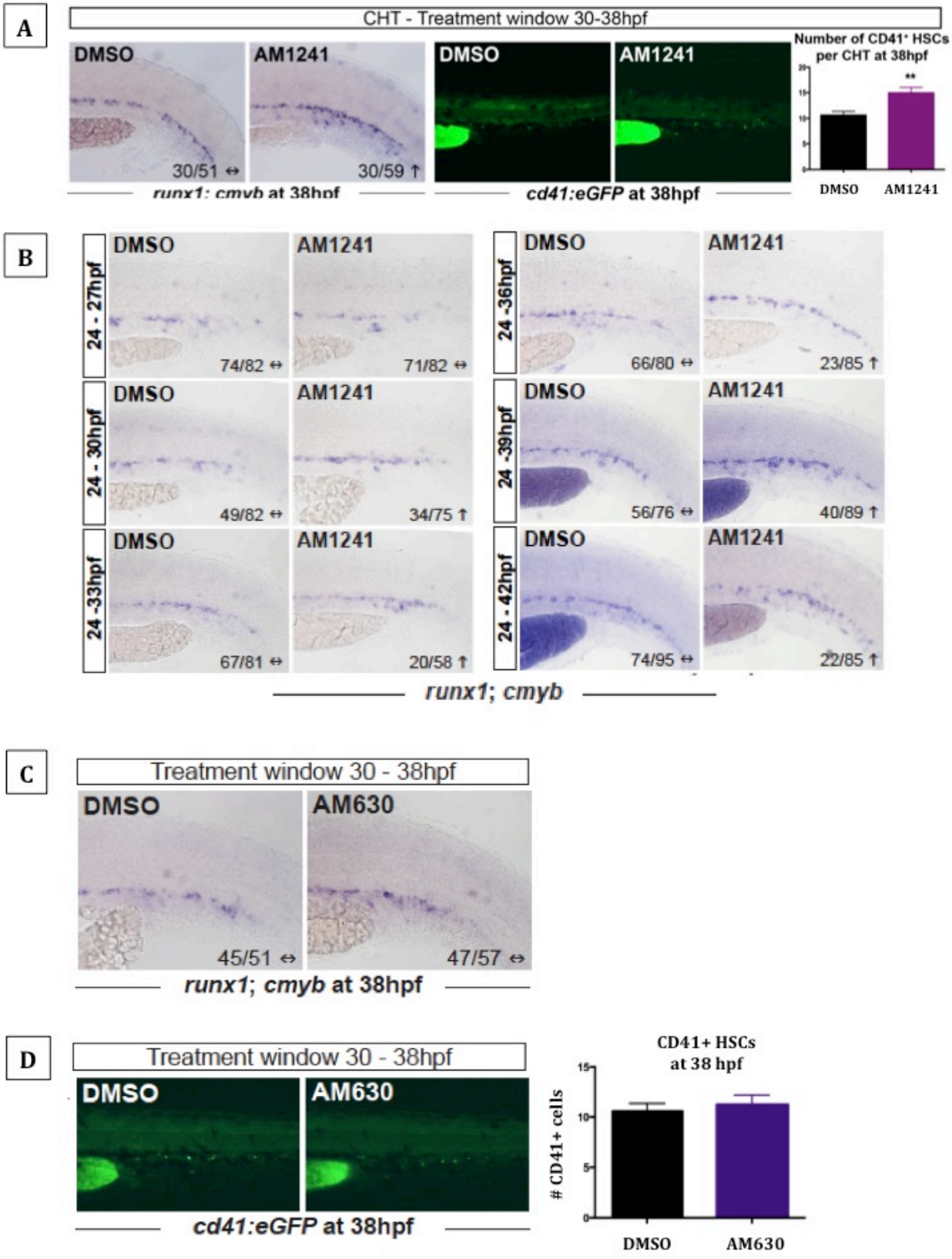
Notably, embryos exposed to AM1241 during the phase of HSPC production (30-38hpf), had no change in AGM *runx1*;*cmyb* expression, however HSPCs robustly increased in the CHT, as confirmed by fluorescence microscopy and absolute CHT HSC counts of *cd41:egfp* embryos (**Figure 4.4 A**). 3-hour interval time-course analysis during CHT colonization (27-42hpf) indicated that in WT embryos *runx1*;*cmyb*$^{+}$ HSPCs appeared in the CHT starting at 33hpf, whereas AM1241-exposed embryos colonized the CHT by 30hpf, with consistently elevated HSPC numbers from 33-39hpf (**Figure 4.4 B**). These data suggested AM1241 enhanced the migration of the newly produced AGM HSPCs toward the CHT. In contrast, embryos exposed to AM630 during HSPC production (30-38hpf) did not exhibit significantly altered *runx1*;*cmyb* expression in the CHT (**Figure 4.4 C**), or numbers of HSCs as confirmed in *cd41:egfp* embryos (**Figure 4.4 D**). Together, these data indicate CNR2-signaling is not essential for migration of newly produced HSPCs to the CHT, but stimulation significantly enhances colonization.

To assess the role of CNR2-signaling in HSPC expansion and/or colonization of secondary hematopoietic niches, embryos were exposed to AM1241 or AM630 from 30hpf-4dpf and *cmyb*$^{+}$ HSPCs were assessed in the CHT, KM and thymus. Interestingly, CHT HSPC number was no longer impacted by AM1241 exposure at 4dpf (**Figure 4.5 A**). In contrast, prolonged AM630 exposure, drastically reduced *cmyb*$^{+}$ HSPCs in the CHT, indicating CNR2-

Figure 4.4

A. Embryos exposed to AM1241 during the phase of HSC production (30-38hpf) exhibited a higher number of *runx1*;*cmyb*$^{+}$ HSPCs in the CHT at 38hpf (DMSO: 30/51= , AM1241: 30/59↑). *In vivo* imaging of *cd41:egfp* embryos showed that CD41^{+} HSCs were increased in the CHT at 38hpf following CNR2-agonist exposure (DMSO: 10.6±0.8, AM1241:15.0±1.1, $p<0.0023$, $n>20$).
B. Embryos exposed to AM1241 during HSC specification and production (from 24hpf) have an earlier onset of runx1;cmyb+ HSPCs in the CHT (from 30hpf) compared to DMSO-treated (33hpf) and exhibit a higher number of HSCs in the CHT up to 39hpf. The number of altered vs. scored embryos is indicated on the figure.
C. Inhibition of CNR2-signaling following AM630 exposure did not affect *runx1;cmyb+* cells in the CHT DMSO: 45/51=, AM630: 47/57=)
D. CNR2-inhibition has no effect on the number of or CD41+ HSPCs (DMSO: 10.6±0.8 CD41+ cells per CHT, AM630: 11.3±0.9, NS, n=20).

Figure 4.4 (Continued)



signaling is required for their expansion during this time window (**Figure 4.5 A**). To determine if this inconsistency was due to enhanced migration to subsequent sites, we examined the KM and thymus: no consistent change was seen in *cmyb*$^{+}$ HSPCs colonizing the KM following AM1241 exposure; in contrast, the appearance of *cmyb*$^{+}$, *rag1*$^{+}$, and *lck* progenitors in the thymus was robustly increased by WISH (**Figure 4.5 B**), implying CNR2-signaling may selectively modulate thymic homing. In agreement with this finding, AM630 exposure from 30hpf-4dpf decreased thymic *cmyb, rag1*, and *lck* staining (**Figure 4.5 B**), together indicating CNR2-signaling regulates HSPC expansion and thymic colonization during definitive hematopoiesis.

CNR2- and PGE2-signaling pathways interact at the level of Cox2 to modulate HSPC development in the AGM region.

As eicosanoid pathways are known to be interconnected (Fowler, 2012), we next explored if CB and PGE2 were coordinately and/or redundantly regulating embryonic HSPC formation. To reveal a collaborative effect, we exposed zebrafish embryos to AM1241 and dmPGE2 alone and in combination from 12-30hpf: following individual treatment, each increased *runx1*$^{+}$ expression (**Figure 4.6 A**) and dual treatment further elevated the percentage of embryos impacted. This effect was quantified by FACS at 36hpf using *cd41:egfp*;*cd45:dsRed* embryos (**Figure 4.6 A**). To determine the mechanism of the interaction, we performed chemical and genetic loss-of-function studies. PGE2 is synthesized from AA through cyclooxygenases Cox1 and Cox2, and prostaglandin E synthase (**Figure 4.6 B**); PGE2 receptors, Ptger2 and Ptger4, mediate effects on HSC development and homeostasis (North et al., 2007). Consistent with prior observations

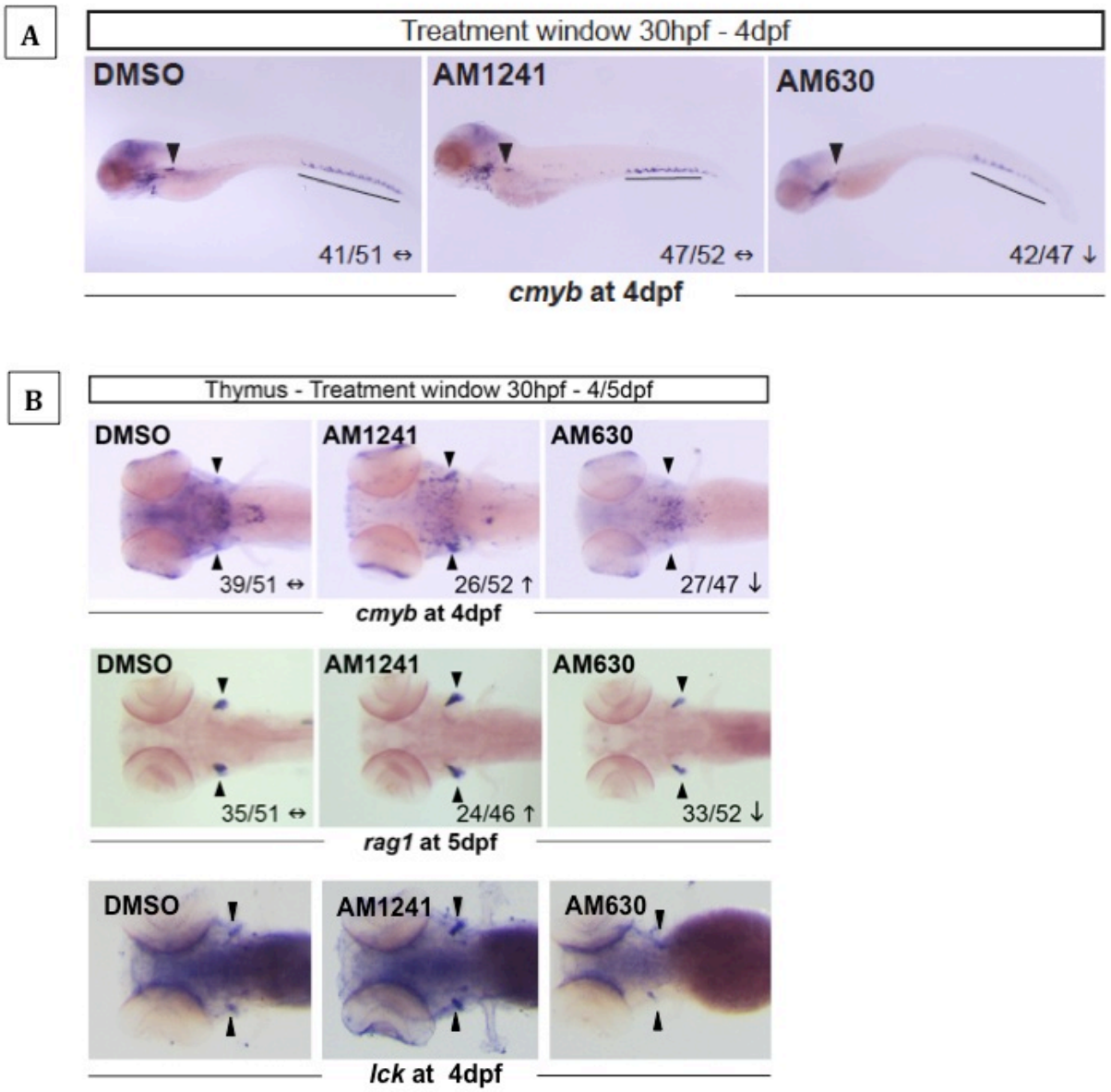


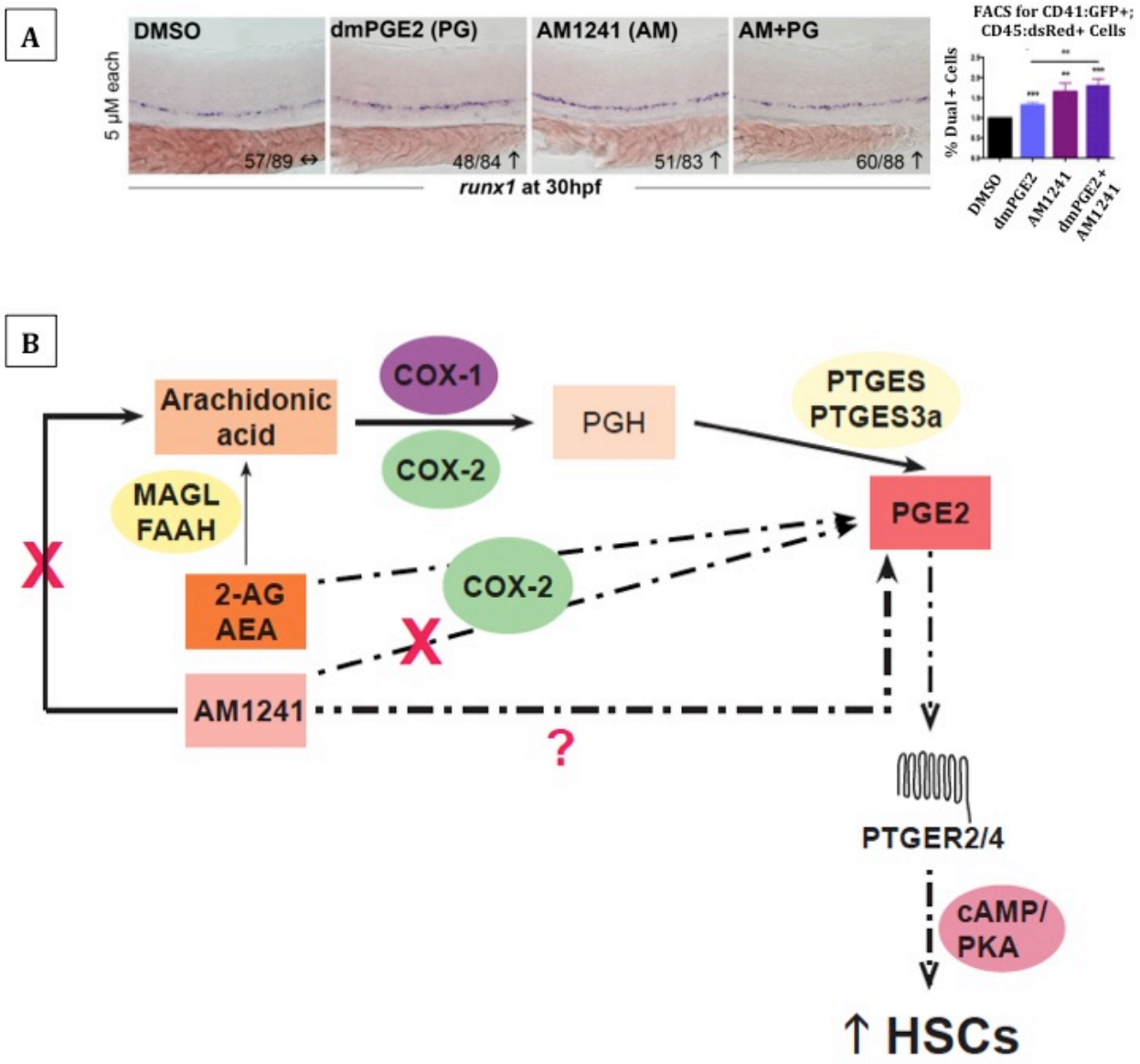
Figure 4.5

A. Embryos exposed to AM1241 from 30hpf to 4dpf exhibited no change in the number of *cmyb+* HSPCs in the CHT (bar) and in the KM (arrowhead) (DMSO: 41/51=, AM1241: 47/52=). However, treatment with AM630 reduced the total number of *cmyb+* HSPCs in these tissues (AM630: 42/47↓).
B. Embryos exposed to AM1241 during HSC production, maturation and expansion (30hpf to 4-5dpf), exhibited a higher number of *cmyb*$^{+}$ and *rag1*$^{+}$ cells in the thymus, whereas AM630 exposure decreased the number of thymic progenitors (arrowheads) (upper panel, *cmyb*, DMSO: 39/51=, AM1241: 26/52↑, AM630:27/47↓; lower panel, *rag1*, DMSO: 35/51=, AM1241: 24/46↑, AM630: 33/52↓). Expression of *lck* was also enhanced after treatment with AM1241 and decreased following AM630 exposure.

Figure 4.6

A. Co-exposure with AM1241 and the stabilized form of PGE2 (dmPGE2) had a collaborative effect on *runx1*$^{+}$ HSC number in the AGM (DMSO: 57/89=, dmPGE2: 48/84↑; AM1241: 51/83↑, dmPGE2+AM1241: 60/88↑). This effect was quantified by FACS using *cd41:gfp*; *cd45:dsRed* dual transgenic line (dmPGE2, 1.32-fold, $p<0.00017$; AM1241, 1.66-fold, $p<0.0043$; AM1241+dmPGE2, 1.82-fold, $p<0.00011$; AM1241 vs. AM1241+dmPGE2, 1.10-fold, NS; dmPGE2 vs. AM1241+dmPGE2, 1.37-fold, $p<0.0097$).
B. PGE2 is synthesized from arachidonic acid (AA) in a two-step process requiring a cyclooxygenase (Cox1 or Cox2) and a PGE2 synthase (Ptges or Ptges3a). PGE2 modulates HSC development through the binding of its receptors Ptger2 and 4 further activating the cAMP/PKA signaling pathway. The endocannabinoids 2-AG and AEA can be metabolized into AA by the MAGL and FAAH, respectively or directly processed by Cox2 into a substrate for PGE2 synthesis. Contrary to the endocannabinoids, synthetic cannabinoids such AM1241 cannot be enzymatically processed and must regulate PGE2 synthesis through an indirect mechanism of action. Heavy and dashed lines represent single- and multi-step processes, respectively.

Figure 4.6 (Continued)



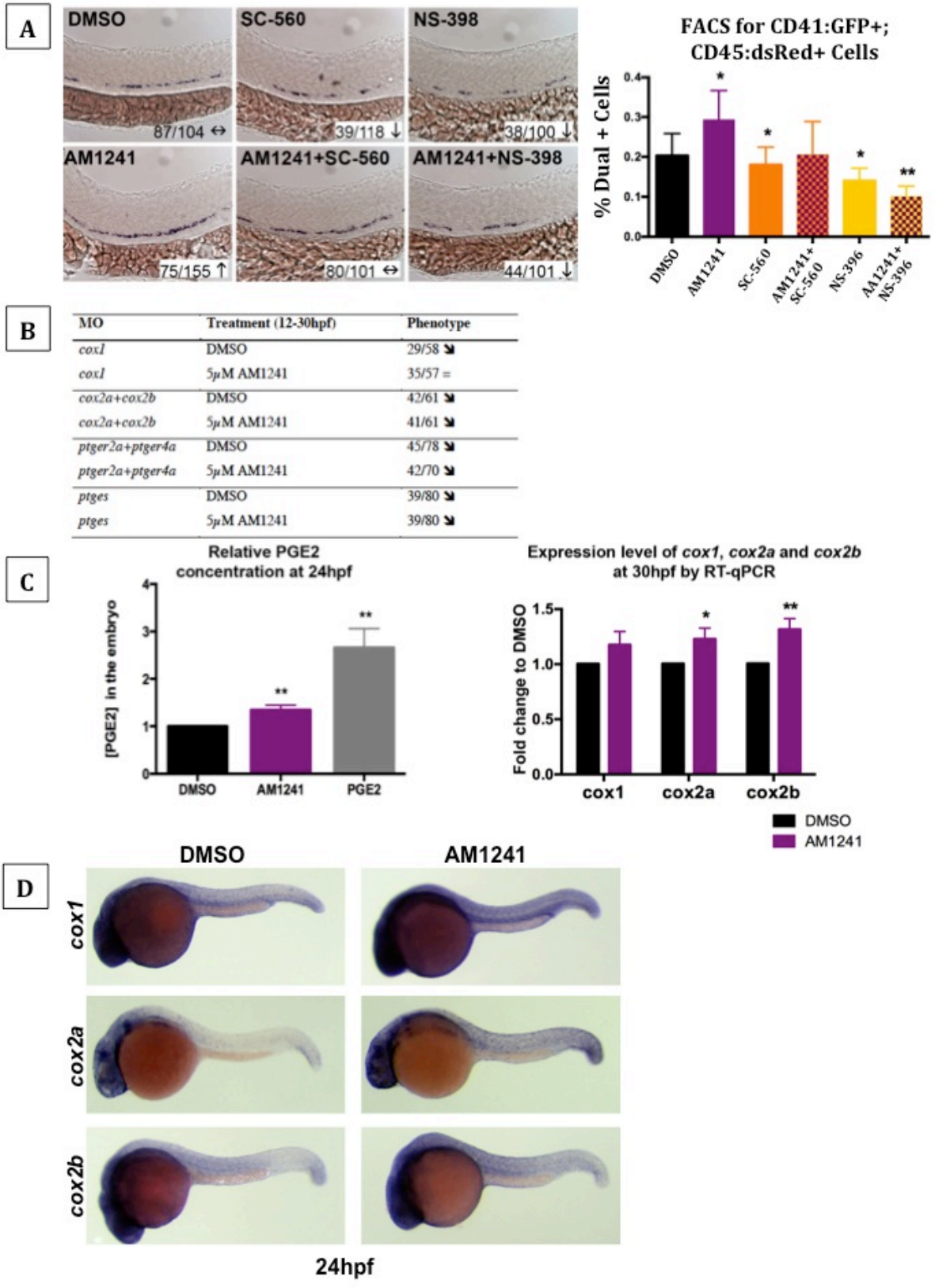
(North et al., 2007), exposure to Cox1- (SC-560) or Cox2-(NS-398) selective inhibitors from 12-30hpf decreased *runx1* expression (**Figure 4.7 A**). Co-treatment with AM1241 partially rescued HSPCs in SC-560- but not NS-398-exposed embryos suggesting Cox2 activity is required for the effect of CNR2 stimulation during niche specification. This result was quantified by *cd41:eGFP;cd45:dsRed* FACS (**Figure 4.7 A**), and further confirmed by MO-knockdown of PGE2 synthesizing enzymes and receptors (**Figure 4.7 B)**: exposure to AM1241 rescued alterations in *runx1* expression resulting from the *cox1* MO, but not that of *cox2*, or *ptger2/4*, indicating PGE2 production by Cox2 was responsible for the CNR2-mediated HSPC phenotype.

Unlike ECs, synthetic derivatives such as AM1241 cannot be enzymatically processed and converted into derivatives of PGE2 (**Figure 4.6 B**), however, CBs can modulate the transcription or activity of Cox2 (Chen et al., 2005; Eichele et al., 2009; Gardner et al., 2003; Hinz et al., 2004; Mestre et al., 2006; Mitchell et al., 2008; Ramer et al., 2003; Slanina and Schweitzer, 2005) to stimulate the production of PGE2 from AA (**Figure 4.6 B**). To test this directly, we measured PGE2-metabolite concentrations using a biochemical assay. While embryos treated with PGE2 showed anticipated increases in PGE2 content (2.66-fold) relative to baseline, exposure to AM1241 likewise significantly (1.35-fold) enriched PGE2-metabolites (**Figure 4.7 C**). Further, by qPCR at 30hpf (**Figure 4.7 C**): whereas *cox1* expression was not statistically increased, both *cox2a* and *cox2b* were significantly upregulated following AM1241 exposure; WISH analysis at 24 and 36 hpf confirmed these observation, showing global upregulation in *cox1* and *cox2a/b* expression in response to

Figure 4.7

A. Epistatic analysis using cyclooxygenase (Cox) 1 and 2 specific inhibitors (SC-560 and NS-398, respectively) showed that CNR2- and PGE2-signaling pathways interacted at the level of Cox2. Treatment with SC-560 and NS-398 decreased *runx1* expression in the AGM; the effect of SC-560 but not NS-398 was rescued by adding AM1241 (DMSO: 87/104=, SC-560: 39/118↓; NS-398: 38/100↓, AM1241: 75/155↑, AM1241+SC-560: 80/101=; AM1241+NS-398: 44/101↓). This effect was quantified by FACS using *cd41:gfp*;*cd45:dsRed* dual transgenic line (AM1241: 1.45-fold, $p<0.028$; SC-560, 0.89-fold, $p<0.044$; NS-398: 0.69-fold, $p<0.017$; AM1241+SC-560: 0.99-fold; AM1241+NS-398: 0.50-fold, $p<0.006$).
B. CNR2- interacts with PGE2-signaling at the level of Cox2. MO knock-down of *cox2* or the downstream effectors of PGE2 synthesis (*ptges*) and signaling (*ptger2a/4a*) blocked the action of AM1241. However, MO knock-down of Cox1 was rescued by AM1241. The table indicates the number of altered vs. scored embryos.
C. Embryos exposed to AM1241 during niche specification (12-24hpf) exhibited an augmented PGE2-signaling. The relative concentration of PGE2 was measured in AM1241- and PGE2- (positive control) treated embryos by a biochemical assay for PGE2-metabolites (n=7; PGE2, 2.66-fold, $p<0.00014$; AM1241, 1.35-fold, $p<0.0044$). qPCR analysis showed that *cox2a* and *cox2b* but not *cox1* were significantly up-regulated at 30hpf following AM1241 exposure during niche specification (*cox1*, 1.17-fold, NS; *cox2a*, 1.22-fold, $p<0.026$; *cox2b*, 1.31-fold, $p<0.004$, n=13).
D. Expression of *cox1*, *cox2a*, and *cox2b* was enhanced after treatment with AM1241 at 24hpf.

Figure 4.7 (Continued)



MO	Treatment (12-30hpf)	Phenotype
cox1	DMSO	29/58 ↘
cox1	5μM AM1241	35/57 =
cox2a+cox2b	DMSO	42/61 ↘
cox2a+cox2b	5μM AM1241	41/61 ↘
ptger2a+ptger4a	DMSO	45/78 ↘
ptger2a+ptger4a	5μM AM1241	42/70 ↘
ptges	DMSO	39/80 ↘
ptges	5μM AM1241	39/80 ↘

CNR2 stimulation (**Figure 4.7D**), supporting CNR2 mediated upregulaton of cox2 induction as the mechanism by which PGE2 levels were enhanced to subsequently influence HSCs**.**

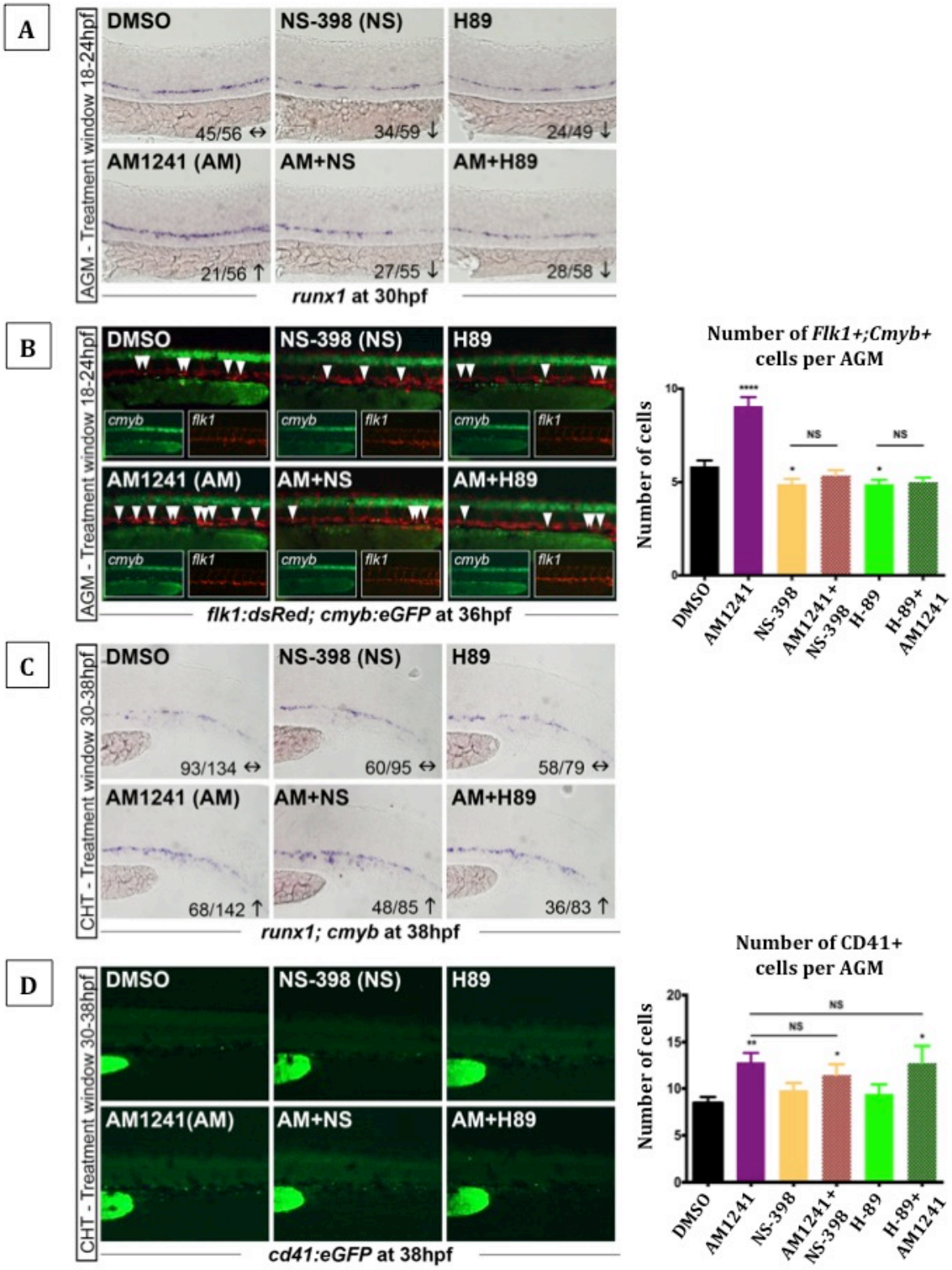
AM1241-mediated increase of PGE2 affects only the AGM during niche specification

To determine if the collaborative interaction between the CNR2- and PGE2-signaling pathways occurred only during a specific developmental time window, embryos were exposed to AM1241 with or without NS-398 for discrete intervals. During niche specification, AM1241 failed to rescue the decreased *runx1* expression mediated by NS-398 in the AGM (**Figure 4.8 A);** however, during HSC production, NS-398 had no effect on *runx1*;*cmyb*$^{+}$ HSPCs in the CHT, nor did it impair AM1241-mediated increases HSPC localization or expansion (**Figure 4.8 C**). To confirm that AM1241 increases PGE2 activity to mediate effects on AGM HSPCs, but not the subsequent colonization phenotypes, we co-exposed embryos to AM1241 and H89, which inhibits PKA activity downstream of PGE2-receptor stimulation (Goessling et al., 2009). As expected, embryos treated with H89 during niche specification had a diminished *runx1*$^{+}$ AGM HSPCs at 30hpf (**Figure 4.8 A**) and co-treatment with AM1241 could not rescue this phenotype. In contrast, when embryos were exposed to AM1241 and/or H89 during HSPC production, H89 was unable to block increases in *runx1*;*cmyb*$^{+}$ HSPCs in the CHT mediated by AM1241 (**Figure 4.8 C**). These findings were confirmed and quantified in the AGM using *flk1:dsRed;cmyb:GFP* embryos at 36hpf and in the CHT using *cd41:eGFP* embryos at 38hpf. Absolute counts of double positive AGM cells showed no effect of CNR2 stimulation if either PGE2 production by Cox2 or signaling via PKA was blocked (**Figure 4.8 B**). Conversely, neither NS-398 nor H89 treatment could significantly blunt the effect of AM1241 when exposure occurred after

Figure 4.8

A. Co-treatment with Cox2 and PKA/cAMP inhibitors (NS-398 and H89, respectively) during niche specification (18-24hpf) abolished AM1241-mediated increase in *runx1* expression in the AGM (DMSO: 45/56=, AM1241: 21/56↑, NS-398: 34/59↓; AM1241+NS-398: 27/55↓, H89: 24/49↓ AM1241+H89: 28/58↓).
B. Counts of dual *positive cmyb:GFP, flk:dsRed* positive cells confirmed results obtained by *runx1* staining.
C. Conversely, blocking the synthesis of PGE2 with the Cox2 inhibitor NS-398 had no effect on the increase in *runx1*;*cmyb*$^{+}$ HSPCs at 38hpf following AM1241 exposure during the phase of HSC production (30-38hpf) (DMSO: 31/51=, NS-398: 30/54=, AM1241: 30/59↑, AM1241+NS-398: 30/48↑). Similar results were obtained when the PKA/cAMP pathway was inhibited with H89 (DMSO: 35/47=, H89: 30/40=; AM1241: 17/40↑; AM1241+H89: 16/41↑).
D. Counts of CD41+ cells indicated that the increase in HSCs after AM1241 treatment at 38hpf is independent of both PGE2 and Cox2.

Figure 4.8 (Continued)



30hpf (**Figure 4.8 D**). Together, these data confirm that AM1241 augments AGM HSPC number via up-regulation of the production and function of PGE2, but later effects on enhanced CHT colonization are independent of PGE2/PKA activity.

AM1241 increases cell proliferation in a Cox2-dependent (AGM) and independent (CHT) manner

During hematopoiesis, PGE2 affects vascular niche development as well as cell proliferation in the AGM (Goessling et al., 2009; Grosser et al., 2002; North et al., 2007). Vasculogenesis was not impacted by AM1241 in *flk1:GFP* embryos nor were markers of vessel identity, *efnb2a* and *flt4* (**Figure 4.9 A-B**). As both PGE2 and CNR2 agonists can regulate cell proliferation and survival (Jiang et al., 2011; North et al., 2007), we sought to determine if AM1241 modulates HSPC expansion in the AGM and/or CHT. During niche specification, AM1241 increased proliferation within the AGM (**Figure 4.10 A**) as delineated by phospho-histone H3 (pH3) in *flk1:*GFP^{+} vessels, whereas NS-398 decreased pH3^{+} cells and blocked the effect of AM1241. Conversely, during HSC production, while AM1241 enhanced the proliferation of *cmyb*:GFP^{+} HSPCs in the CHT as delineated by *flk1:*dsRed co-expression (**Figure 4.10 B**), Cox2 inhibition had no effect on its own nor did it prevent the pro-proliferative action of AM1241. These data indicate a dual mechanism of action for CNR2 stimulation, the first involving PGE2 production and function in the AGM, but the latter, early CHT colonization and HSPC expansion, enacted by an alternative method.

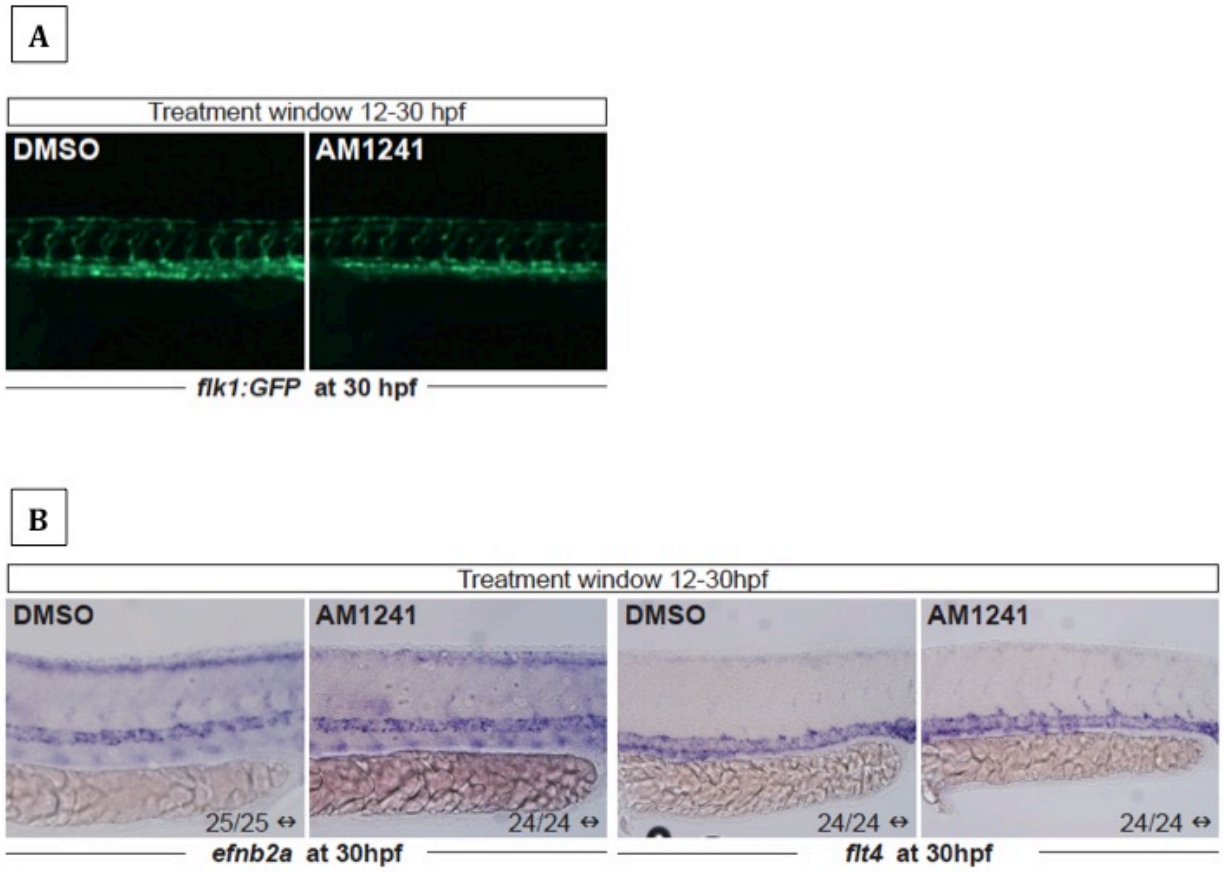


Figure 4.9

A. The vascular niche was not affected following AM1241 exposure (12-30hpf) as assessed by *flk1:GFP* at 30hpf (n>20).
B. The arterial (*efnb2a*) and venous (*flt4*) identities were correctly established following AM1241 treatment (12-30hpf) as determined by in situ hybridization at 30hpf (n>24).

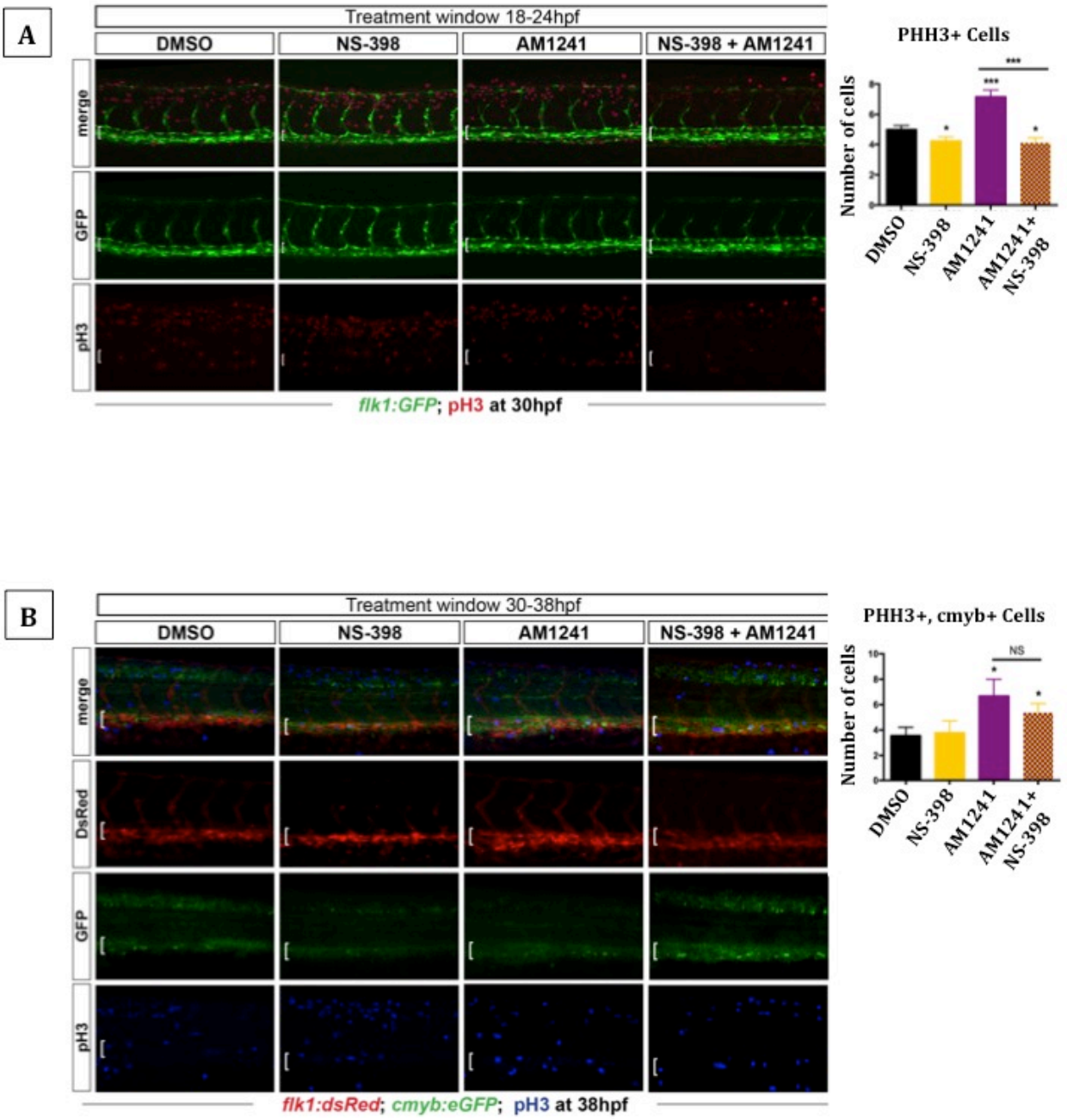


Figure 4.10

A. Embryos exposed to AM1241 during niche specification (18-24hpf) exhibited a higher number of pH3$^+$ cells in the AGM region (labeled with *flk1:*GFP) at 30hpf. This effect was blocked when the activity of Cox2 was inhibited by NS-398 (DMSO: 5.0±0.27; NS-398: 4.22±0.28, $p<0.031$; AM1241: 7.15±0.45, $p<0.0004$; AM1241+NS398: 4.09±0.34, $p<0.025$ vs. DMSO, $p<0.0001$ vs. AM1241, $n\geq9$).

B. Embryos exposed to AM1241 during HSC production (30-38hpf) exhibited a higher number of *cmyb*:GFP$^+$;pH3$^+$ cells in the CHT (labeled with *flk1:*dsRed2) at 38hpf. This effect was independent of the activity of Cox2. pH3$^+$ cells in the CHT region were manually counted (DMSO: 3.55±0.57; NS-398: 3.78±0.94, NS; AM1241: 6.67±1.33, $p<0.027$; AM1241+NS398: 5.36±0.72, $p<0.043$, $n\geq9$

The effect of AM1241 in the CHT and thymus is mediated through the P-selectin pathway

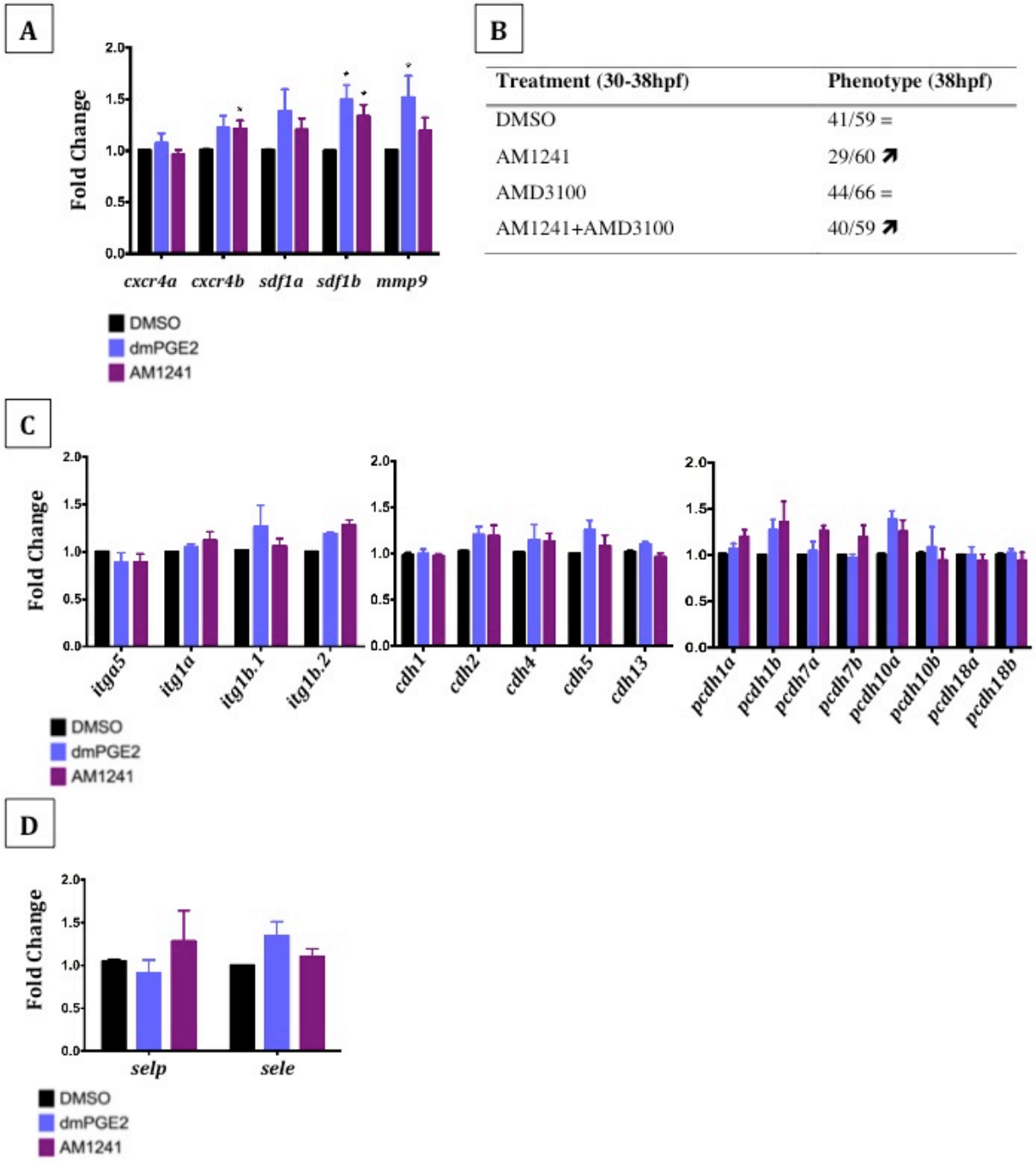
PGE2 is thought to increase HSPC homing and retention to the BM by regulating hematopoietic growth factors and cytokines (Goessling et al., 2011), including CXCR4 (Hoggatt et al., 2009). CNR2-signaling also modulates adult blood cell migration (Hoggatt and Pelus, 2010; Jordà et al., 2002; Joseph et al., 2004; Kishimoto et al., 2005; Liu et al., 2013; Oka et al., 2004; Valk et al., 1997; Valk and Delwel, 1998). As the effect of AM1241 on CHT colonization is independent of PGE2, we searched for adhesion molecules that were differentially regulated by each eicosanoid during HSC production (30-36hpf). Although dmPGE2 and CNR2-agonists are reported to have opposite effects on CXCL12/CXCR4 *in vitro* (Ghosh et al., 2006; Hoggatt et al., 2009), both *cxcr4b* and *cxcl12b* were significantly up-regulated following either treatment in the embryo (**Figure 4.11 A**). Similarly, MMP9 expression reportedly exhibits opposing responses to PGE2- and CNR2-signaling (Adhikary et al., 2012; Yen et al., 2011); in the embryo, while dmPGE2 increased *mmp9* expression, it was unchanged after AM1241 treatment, making it an unlikely candidate for CNR2-stimulated CHT colonization (**Figure 4.11 A**). Epistatic experiments using AMD3100 (CXCR4 antagonist) and SB-3CT (MMP2/9 inhibitor), and *cxcr4*/*cxcl12* knockdown studies confirmed the expression analysis (**Figure 4.11 B** and *data not shown*).

We next assessed adhesion factors expressed in the vasculature of the developing zebrafish embryo from the Cadherin, Protocadherin, and Integrin families (zfin.org, **Figure 4.11 C**): by qPCR, none exhibited statistically differential regulation following PGE2 or AM1241 treatment. *p-selectin* (*selp*) and its ligand, *p-selectin glycoprotein ligand-1* (*psgl-1*), are

Figure 4.11

A. qPCR analysis of embryos (>25/condition, 4 biological replicates per set) treated during HSC production (30-36hpf) showed a significant change of expression for *cxcr4b* following AM1241 exposure (1.20 fold, $p<0.044$) and *sdf1b* following dmPGE2 and AM1241 treatment (dmPGE2 :1.49 fold, $p<0.015$; AM1241 : 1.34 fold, $p<0.025$). *mmp9* expression was up-regulated following dmPGE2 exposure during HSC production (30-36hpf) but remained unchanged after AM1241 treatment (dmPGE2 : 1.31 fold, $p<10\text{-}4$; AM1241 : 1.19 fold, NS).
B. Blockade of CXCR4 signaling with AMD3100 did not affect the ability of AM1241 to increase runx1;cmyb+ HSPCs in the CHT. The table indicates the number of altered vs.scored embryos.
C. qPCR analysis of embryos treated during HSC production (30-36hpf) did not show a different effect of dmPGE2 and AM1241 treatment on the expression level of integrins (*itga5, itgb1a, itgb1b.1, itgb1b.2*) cadherins (c*adh1, cadh2, cadh4, cadh5, cadh13*) and protocadherins (*pcdh1a/b, pcdh7a/b, pcdh10a/b, pcdh18a/b*) involved in the HSPC trafficking in the BM.
D. *p-selectin* (*selp*) and *e-selectin* (*sele*) expression was not significantly altered in dmPGE2-and AM1241-treated embryos.

Figure 4.11 (Continued)



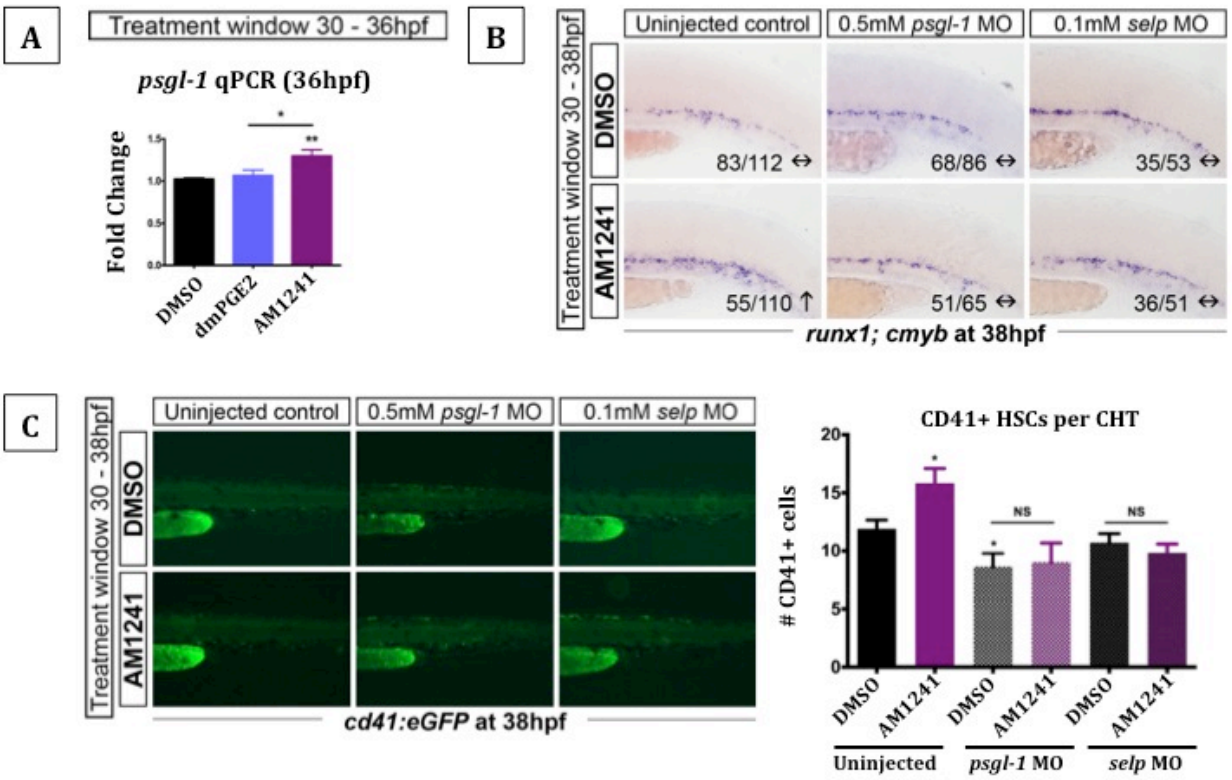


Figure 4.12

A. qPCR analysis showed that the expression level of *p-selectin ligand* (*psgl-1*) was significantly up-regulated during HSC production by AM1241 (30-36hpf) (n=10, dmPGE2, 1.04-fold, NS; AM1241, 1.26 fold, $p<0.0034$, dmPGE2 vs. AM1241 $p<0.043$).

B. Knock-down of *psgl-1* and *selp* by MO injection didn't affect *runx1*;*cmyb*$^{+}$ HSC number in the CHT but prevented AM1241 from increasing HSC number during HSC production (Uninjected/DMSO: 83/112=, *psgl-1* MO/DMSO: 68/86=, *selp* MO/DMSO: 35/53=, Uninjected/AM1241: 55/110↑, *psgl-1* MO/AM1241: 51/65=, *selp* MO/AM1241: 36/51=).

C. Counts of *CD41+* cells confirmed that knockdown of either *psgl* or *selp* blocked the AM1241-stimulated increase in HSCs.

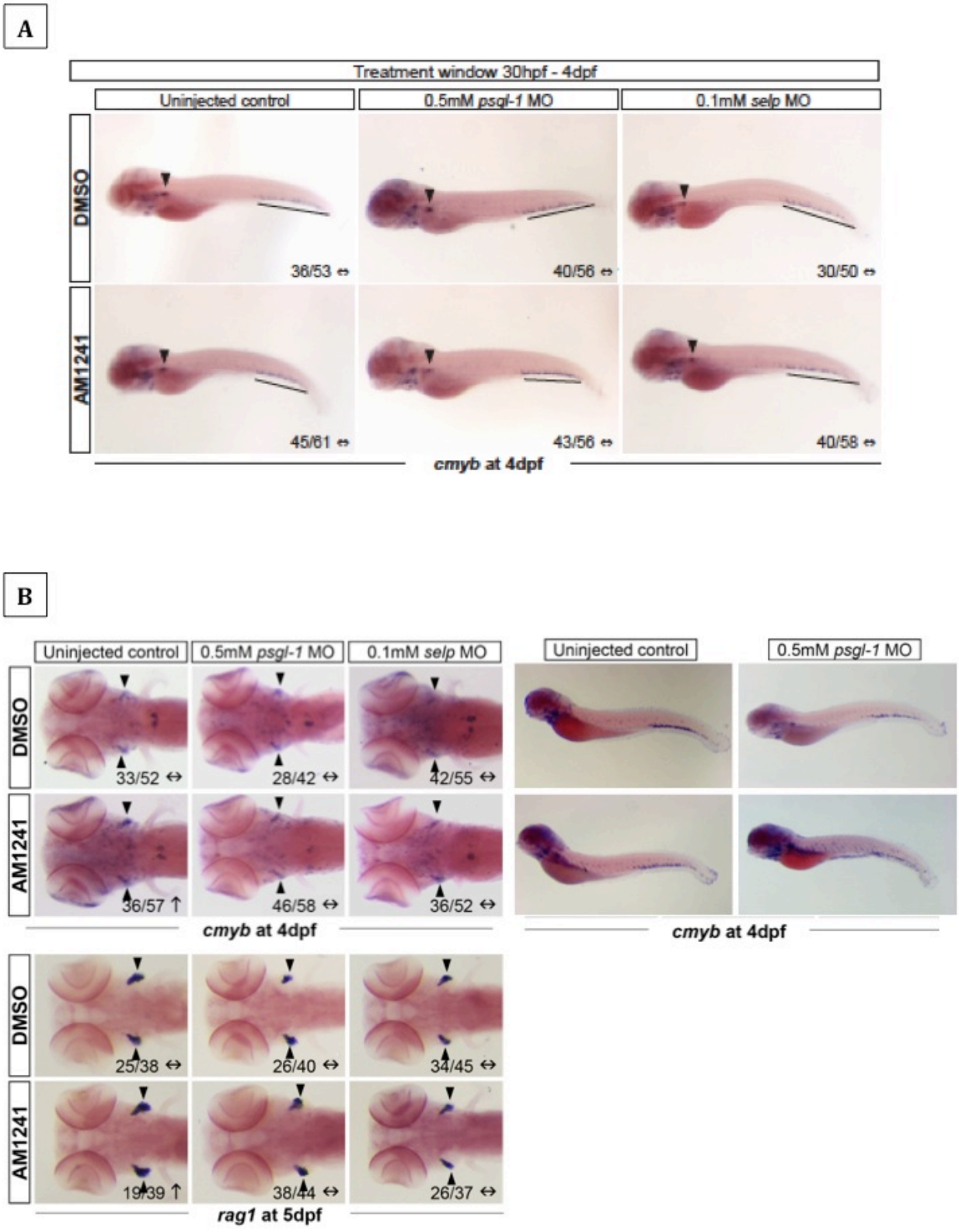
expressed in the vasculature from 18hpf (Sun et al., 2012; 2010); while neither *selp* nor *e-selectin* (*sele*) showed statistically differential regulation (**Figure 4.11 D**), *psgl-1* expression was significantly up-regulated following AM1241 treatment but not dmPGE2 (**Figure 4.12 A**). To determine if this pathway enables the effect of AM1241 in CHT colonization, we knocked down *selp* and *psgl-1*. At 38hpf, *runx1*;*cmyb* expression in the CHT was not significantly altered in *selp* or *psgl-1* morphants (**Figure 4.12 B**). However, following *selp* or *psgl-1* knockdown, AM1241 no longer increased HSPC number in the CHT (**Figure 4.12 B**); these results were confirmed and quantified using *cd41:eGFP* embryos and together indicate that the effect of AM1241 on CHT colonization occurs via an up-regulation of P-selectin activity (**Figure 4.12 C**).

As the number of thymic progenitors also increased following AM1241 exposure (**Figure 4.5 A**), we next sought to determine if the P-selectin pathway is likewise involved in this process. After MO-mediated knock-down of *selp* and *psgl-1*, the number of *cmyb*$^{+}$ cells in the CHT and KM at 4dpf was not significantly affected (**Figure 4.13 A**), however the number of thymic HSPCs as well as *rag1* and *lck* lymphoid progenitors appeared reduced (**Figure 4.13 B**). Likewise, while total *cmyb* expression increased, AM1241 failed to increase the colonization of thymic HSPCs, or appearance of *rag1* and *lck* progenitors in morphant embryos (**Figure 4.13 B**). Analysis of AM1241 treated *psgl-1* MO morphants revealed a marked increase in the distribution of *cmyb* expressing cells throughout the embryo, which was not seen in AM1241 or DMSO controls; *psgl-1* MO knockdown alone appeared to likewise change the overall distribution of *cmyb*+ cells, likely explaining the lower thymic *rag2* and *lck* populations. Together, these data indicate that after the window

Figure 4.13

A. Embryos exposed to AM1241 from 30hpf to 4dpf exhibited no change in *cmyb+* HSPCs in the CHT (bar) and KM (arrowhead) (Uninjected/DMSO: 36/53=, *psgl-1* MO/ DMSO: 40/56=, *selp* MO/DMSO: 30/50=, Uninjected/AM1241: 45/61=, *psgl-1* MO/ AM1241: 43/56=, *selp* MO/AM1241: 40/58=).
B. Knock-down of *psgl-1* and *selp* by MO injection didn't affect $cmyb^{+}$ and $rag1^{+}$ thymic progenitors but prevented AM1241 from increasing their number in the thymus (arrowhead) (upper panel, *cmyb*, Uninjected/DMSO: 33/52=, *psgl-1* MO/DMSO, 28/42=; *selp* MO/DMSO, 42/55=, Uninjected/AM1241: 36/57↑, *psgl-1* MO/AM1241, 46/58=; *selp* MO/AM1241, 36/52=; lower panel, *rag1*, Uninjected/DMSO: 25/38=, *psgl-1* MO/DMSO, 26/40=; *selp* MO/DMSO, 34/45=, Uninjected/AM1241: 19/39↑, *psgl-1* MO/AM1241, 38/44=; *selp* MO/AM1241, 26/37=). Images on the right display whole-embryo expression of *cmyb*.

Figure 4.13 (Continued)



of HSC specification, CNR2-signaling modulates HSC expansion and migration to secondary niches via up-regulation of the P-selectin pathway.

Discussion

HSC specification and production from hemogenic endothelium is a complex and multi-step process requiring the combined and sequential action of multiple pathways (Clements and Traver, 2013). The mechanisms underlying subsequent niche colonization to allow HSC maturation, expansion and homing to adult hematopoietic sites are less well characterized and are likely to require cytokines, ECM modifiers and adhesion molecules secreted from the niche environment (Cao et al., 2013). The orchestration of these different processes is exquisitely timed and presumably tightly regulated to ensure the production of sufficient numbers of mature HSCs, while simultaneously allowing a subset to differentiate to the various effector lineages; as such, hematopoiesis must involve multiple overlapping, and perhaps even redundant pathways, with mechanisms for feedback and cross-regulatory control to achieve the appropriate balance. Here, we present evidence that CNR2-signaling can modify HSPC production and proliferation within the AGM, as well as migration to the CHT and the thymus, and subsequent HSC expansion (**Figure 4.14**); however, we additionally discovered hematopoiesis continues, albeit less effectively, in its absence. Together, these data indicate that CNR2-signaling is a modifier of the multiple steps of HSC regulation during embryogenesis that can potentiate the effects of other known or still unknown regulators.

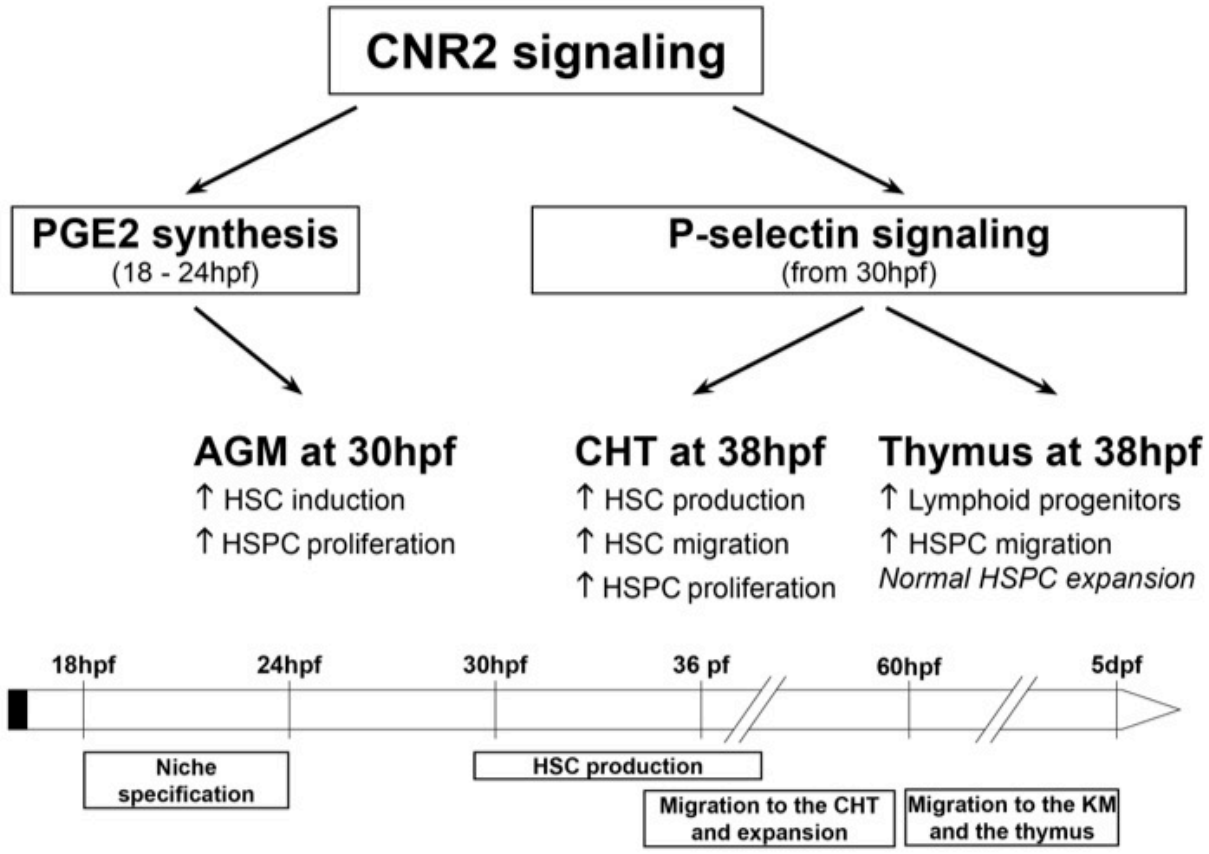


Figure 4.14

During niche specification, CNR2-signaling heightens the levels of PGE2 through the up-regulation of Cox2. This leads to an increase in HSC induction in the AGM at 30hpf. This effect is accompanied by an increased cell proliferation that is Cox2-dependent. During HSC production, CNR2-signaling increases the expression of P-selectin ligand (*psgl-1*) leading to a faster migration of HSCs to the CHT. This effect is accompanied by an augmented cell proliferation and is independent of PGE2 synthesis.

Eicosanoids, including PGE2 and CBs, have emerged as potent modulators of HSC formation and function in adult vertebrates (Hoggatt and Pelus, 2010; Hoggatt et al., 2009; Jiang et al., 2011; North et al., 2007). Here, we revealed novel roles for CNR2-signaling during embryogenesis, impacting several aspects of HSPC development. While exposure to CNR2-agonists during AGM specification increased HSPC number by enhancing PGE2 levels in a Cox2-dependent manner, treatment during the phase of HSC production instead accelerated the colonization and expansion of *cmyb*$^{+}$ and *cd41*$^{+}$ cells in the CHT, and later the *lck*/*rag1*$^{+}$ population in the thymus, via the P-selectin pathway. In the adult, CNR2-signaling modulates HSC proliferation and survival, as well as mobilization from the BM niche (Hoggatt and Pelus, 2010; Jiang et al., 2011). Factors regulating BM mobilization and retention, such as CXCR4/CXCL12 and E-/P-Selectin, can likewise regulate cell proliferation (Eto et al., 2005; Lévesque et al., 1999; Mo et al., 2013; Winkler et al., 2012). Our attempt to determine if HSPC elevation following CNR2 stimulation was a consequence of the earlier arrival of HSPCs in the CHT, allowing more time to expand, or a direct pro-proliferative effect on HSPCs, was complicated by the fact that both functions are contemporaneous. However, while colonization of secondary niches did occur in the absence of CNR2-signaling, the drastic reduction in *cmyb*$^{+}$ HSPCs at 4dpf in all hematopoietic tissues mediated by inhibition of CNR2-activity implies regulation of HSPC proliferation is not simply a secondary outcome.

While often considered directly opposing actions, HSC mobilization and homing or retention are not mirror images, but different processes involving overlapping and distinct

mechanisms of action (Lévesque et al., 2010). In this study, we show CNR2 modulation, through the action of its downstream effector P-selectin, affects HSCs migrating from the AGM to CHT, and CHT to thymus but *not* to the KM, suggesting its role in HSPC regulation is more complex than simply stimulating egress and/or is influenced by or redundant with other factors. The P-selectin pathway influences homing of the adult lymphoid progenitors to the thymus by modulating extravasation from the blood vessels (Rossi et al., 2005); P-selectin is expressed on thymic endothelium (Rossi et al., 2005) and PSGL-1 expression is gradually up-regulated as HSCs mature to CLPs (Sultana et al., 2012). However, the mechanisms controlling the seeding of the embryonic thymus, including a role for P-selectin activity, remain largely unknown (Bajoghli et al., 2009; Bleul and Boehm, 2000; Calderón and Boehm, 2011; Liu et al., 2006; 2005). While both CNR1 and CNR2 are highly expressed in the murine fetal thymus (Lombard et al., 2011), perinatal exposure to D_9-THC, the bio-active component of cannabis, leads to profound thymic atrophy and induction of T-cell apoptosis (Lombard et al., 2011). These data appear inconsistent with our analyses, where CNR2-agonists increased the number of thymic progenitors and the absence of CNR2-signaling drastically reduced them; however, this discrepancy may come from the window of exposure, as we were assessing the role of CNR2-signaling from the production of AGM HSCs and the fact that we examined *cmyb*$^+$ or *lck/rag1*$^+$ T-cell progenitors, an earlier subpopulation included within the double-negative CD4/ CD8 fraction, but not the later thymocytic stages (Lombard et al., 2011). Alternatively, the enhanced colonization we see following AM1241 exposure may be specific to the action of CNR2; other studies have primarily focused on endogenous and plant-derived CBs, which exhibit a higher affinity for CNR1. *In vitro*, CNR2 stimulation can promote or inhibit T-cell migration and proliferation

depending on the choice of agonist and dose utilized (Basu and Dittel, 2011), highlighting that context and/or treatment regimen will be of critical importance to outcome, and again implying CBs function as modifying, rather than required, factors regulating HSPCs.

In this study, we identified PGE2 and P-selectin as targets of CNR2-signaling in distinct phases of HSC development. The interplay between bioactive lipids, which can derive from common precursors, intra-convert, as well as transcriptionally activate the enzymatic machinery of related pathways, is a previously underappreciated mechanism to modulate their individual regulatory outcomes; one may speculate that such interactions may be involved in the pleiotropic effect of CNR2-signaling on HSC development. PGE2 is an established regulator of HSCs across vertebrate species and a promising therapeutic candidate currently being examined in phase II clinical trials to increase efficacy of umbilical cord blood transplantation(Cutler et al., 2013). Similar to other contexts (Chen et al., 2005; Eichele et al., 2009; Gardner et al., 2003; Hinz et al., 2004; Mestre et al., 2006; Mitchell et al., 2008; Ramer et al., 2003; Slanina and Schweitzer, 2005), here we show CNR2-signaling directly modulates PGE2 production in a Cox2-dependent manner, which in turn increases HSPC number. The effect of CB-signaling on P-selectin expression remains controversial, as it is able to increase or decrease *P-selectin/PSGL-1* expression depending on the model (Burt and Chu, 2008; Deusch et al., 2004; Matos et al., 2013; Shinohara et al., 2012; Xu et al., 2007; Zhao et al., 2010); these discrepancies may reflect an indirect mechanism of regulation by CB. Interestingly, thymic levels of the bioactive phospholipid S1P vary in the same manner as that of P-selectin (Gossens et al., 2009). S1P regulates lymphocyte trafficking (Schwab et al., 2005) as well as HSC mobilization to the PB (Juarez

et al., 2012). CB- and S1P-signaling can interact at multiple levels: AEA increases the phosphorylation of S1P (Sim-Selley et al., 2009), and CNR1-stimulation enhances the biosynthesis of ceramide, the substrate for S1P (Galve-Roperh et al., 2000; Gustafsson et al., 2009); conversely, sphingosine can bind to CNR1 as an antagonist (Selley et al., 2013), suggesting feedback regulation between these two lipid signaling pathways. It will be interesting to determine whether just as CNR2-signaling modulates AGM HSPCs through cross-regulatory effects on PGE2 levels, if CHT and thymus colonization occurs via enhanced biosynthesis or antagonistic regulation of S1P or other lipid mediators.

Demand-driven hematopoiesis, initiated by inflammatory feedback loops, is well characterized to stimulate HSC mobilization, proliferation and differentiation to provide an adequate response to infection or injury. In the adult, HSCs and more committed downstream progenitors, express receptors for most of the pro-inflammatory molecules (Takizawa et al., 2012), however, a similar role for these factors during embryogenesis remains to be described. Here, we show inflammatory intermediates, CBs and PGE2, can cooperate to modulate select aspects of HSC function during embryogenesis. As we previously demonstrated that factors within this class, such as PGE2 (Goessling et al., 2009; North et al., 2007) or nitric oxide (North et al., 2009) are active in the embryo and necessary for the proper development of the HSCs, it will be interesting to determine if other inflammatory modulators likewise participate in HSPC regulation from the earliest stages of hematopoietic development. Together, the data presented here reveal a novel role of CNR2-signaling as a modifier of HSC production and function during embryogenesis.

Materials and Methods

Zebrafish husbandry

Zebrafish were maintained according to IACUC-approved protocols. Specific lines and references are in **Appendix II**.

Chemical treatments and evaluation

Zebrafish embryos were exposed to chemicals at the following doses in E3 water in multi-well plates from 5-somites (12hpf) until 36hpf, unless otherwise noted: 2-AG (5 μM), AEA (5 μM), ACEA (5 μM), AM1241 (5-10 μM), JWH015 (5-10 μM), AM630 (5-10 μM), dmPGE2 (5 μM), PGE2 (5 μM), SC-560 (10 μM), NS-398 (10 μM), AMD3100 (10 μM). *In situ* hybridization was performed as previously described (Harris et al., 2013). Phenotype distribution is summarized as: #-altered/#-scored per treatment; >3 independent experiments were conducted per analysis. FACS was previously described (North et al., 2007). For immunohistochemistry, embryos were fixed in 4% PFA, and dehydrated overnight in MeOH at -20ºC. After rehydration and washes in PBS 0.1% Tween20 (PBT), embryos were incubated in pre-chilled acetone at -20ºC for 20min and rinsed in dH2O for 5min at RT before proteinase K digestion (10μg/mL, 6-12min for 30-38hpf). Primary antibodies [α-phospho-histone H3 (06-570; Millipore) α-phospho-histone H3 (ab14955; Abcam); α-GFP (MAB3580; Millipore); α-GFP (SC-9996; Santa Cruz); α-dsRed (632496; Contech); Goat α-rabbit IgG (Invitrogen); Goat α-mouse IgG (Invitrogen)] were incubated overnight in Block (PBT, 2% BSA, 2% sheep serum), PBT washed, and visualized as indicated below after secondary antibody staining (2hrs, RT).

Morpholino injection

MOs (**Appendix II**; GeneTools) were injected as described previously (North et al., 2009).

Fluorescent microscopy

Fluorescent reporter embryos were treated as indicated above and imaged as previously described (North et al., 2007). For IHC, embryos were mounted in Fluoromount-G (Southern Biotech) and imaged using Zeiss LSM 510 Meta 2-Photon or 700 Laser Scanning Confocal Microscopes.

Quantitative RT-PCR

qPCR was performed on cDNA isolated from pooled embryos at timepoints indicated (n=25/variable; primers: **Appendix II**) using ABI PRISM 7900HT (Invitrogen). Fold-changes were calculated using the ddCt method with *tbp* as the reference gene.

PGE2 metabolite measurement assay

PGE2 metabolite measurement was performed on lysates from 30 disaggregated embryos incubated with 5μM AM2141 or 5μM PGE2 between 12-24hpf according to manufacturer's protocol (Cayman Chemical).

Appendix II:

Supplemental Methods and Reagents

Morpholino sequences

Gene	Morpholino	Reference:
esr1	5’ AGGAAGGTTCCTCCAGGGCTTCTCT 3’	(Pang and Thomas, 2010)
esr2a	5’ ACATGGTGAAGGCGGATGAGTTCAG 3’	(Froehlicher et al., 2009)
esr2b	5’ AGCTCATGCTGGAGAACACAAGAGA 3’ -or- 5’ TTGACCATGAGCATTACCTTGAATG 3’	*upper:* W. Goessling *(unpublished)*; *lower:* (Griffin et al., 2013)
cnr1	5’ GTGCTATCAACAACATACCTTTGTG 3’	*Unpublished*
cnr2	5’ GCCATGAAACAAACAGTACCTGTGG 3’	*Unpublished*
cox1	5’ TCAGCAAAAAGTTACACTCTCTCAT 3’	(North et al., 2007)
cox2a	5’ AACCAGTTTATTCATTCCAGAAGTG 3’	(North et al., 2007)
cox2b	5’ AGGCTTACCTCCTGTGCAAACCACG 3’	(Yeh et al., 2009)
ptges	5’ GTTTTGTGCTCTTACCTCCTACAGC 3’	(North et al., 2007)
ptger2a	5’ ACTGTCAATACAGGTCCCATTTTC 3’	(North et al., 2007)
ptger4a	5’ CACGGTGGGCTCCATGCTGCTGCTG 3’	(North et al., 2007)
selp	5’ TTTGTAAGCCACCATCGCCGCCATC 3’	*Unpublished*
psgl-1	5’ CATTGACTGATAAACACAGTGGCGT 3’	*Unpublished*

qPCR Primers

Gene	Forward	Reverse
18s	TCGCTAGTTGGCATCGTTTAT	CGGAGGTTCGAAGACGATCA
tbp	CGGTGGATCCTGCGAATTA	TGACAGGTTATGAAGCAAAACAACA
runx1	CGTCTTCACAAACCCTCCTCAA	GCTTTACTGCTTCATCCGGCT
cmyb	TGATGCTTCCCAACACAGAG	TTCAGAGGGAATCGTCTGCT
flk1	CGAACGTGAAGTGACATACGG	CCCTCTACCAAACCATGTGAAA
vegfAa	AGAAAGAAAACCACTGTGAG	AGGAATGTTCTTCCTTAGGT
flt4	ATCATCTGGCTGAAGGATGG	AGGACACGCTCTTCTCTCCA
ephrinB2	CAAGGACAGCAAATCGAATG	TGAGCCAATGACTGATGAGG
cyclinD1	GGAACTGCTGGCGCTTAAATA	GACTTGCGAGAGGAAGTTGG
c-myc	TGACTGTGGAAAAGCGACAG	GCTGCTGTTGATGCTGTGAT
gata2	ACAACGTCAACAGGCCACTGA	TCGAAACCCTCACCAGATCGT
notch3	GCATTGACCGACCTAATGGA	TGCTCTCACACAGTCTTCCTTC
smyhc1	TGCCAAGACCATCAGAAATG	CACACCAAAGTGAATTCGGATA
prdm1	ATCGTGGCCTGAACCACTAC	GAGGAGGTACCAAGCTGCTG
ptch2	GTTACTGCCACGCCGCTTTTG	CTGACTCCTCTCCTTGCTTCT
gli1	ACCACTACGACAACACCAGCAACC	TTCACAGACTGACCACCAGAGAGC
deltaC	CGCAGAAACCTCTGACCAGT	CAGTCCTCACTGATAGCGAGTC
tbx20	AGATTGACAGCAACCCGTTT	TGCTGAATGTCCTTCTTCTCC
dusp5	ACTTTGTCGCACTTGACGAG	TGGGTTTTCATGATGTACGC
dll4	TGGCCAGTTATCCTGTCTCC	CTCACTGCATCCCTCCAGAC
sclab	GGAGATGCGGAACAGTATGG	GAAGGCACCGTTCACATTCT
tie2	TGGAGGAGCGCAAGACATATG	CATTATTGCTGCAGTGCATGAG
cyp19a1a	AGAACGTCAGGCAGTGTGTG	ACCGGGTGAAAACGTAGAGA
srd5a	TACACACCCCTGCTTCATCA	CAGCCCATTCCACAATCTCT
vtg1	ACTACCAACTGGCTGCTTAC	ACCATCGGCACAGATCTTC
cox1	AGGGTCATCGTTGAATCGAG	ATTTCCACCATGCTTTCACC
cox2a	CCAGACAGATGCGCTATCAA	GAGCTCCCATTTCCACCATA
cox2b	AGGGCGTAATGTAGCACCAG	CAGCATAAAGCTCCACAGCA
psgl-1	TCCAGTGCAGACCGTTAATG	GTTGGGTGTGCAAACTAATC
selp	TCGGGCATACTACTGGATTG	GGTTATTCGGTTCATTTGTCG
sele	TCGCTCTTCAAAGCAACAGA	TCTTATAAGCCTGCGGTGGA
cxcr4a	GCTGGAGACTGAAGGAGCTG	CCCGATAAGACCCAAAACAA
cxcr4b	TGGAGACTTATTGCGCCTTT	CAAGCACCACAAGTCCATTG
cxcl12a	CACAGTCCCACAGAGAAGCA	GGGCTGTCAGATTTCCTTGT
cxcl12b	GGAGCATCCGAGAGATCAAG	CGCACATCCTCGTCTGTTTA
mmp9	TCATGATCTCTGCGAAGTGG	TTGCCTTTTCCTCTCTGCAT
itga5	TGGCGATTCTAGCTGGTCTT	TTCCTCCGCCGTCTACTCTA
itgb1a	GGTGTAGTGGCAGGAATCGT	GGCCATTATTTGCCTTCGTA
itgb1b	GTCTGCAGCAACAATGGAGA	AGAGGGCAGTCACAAGCACT
itgb1b.2	AAAAGAGAAAACCCGGAGGA	ACATTCACAGATGCCGTGTC
cdh1	AGCAGGGATTCCACTGTTTG	CCTCTGTGCAGGACACTCAA

qPCR Primers (Continued)

Gene	Forward	Reverse
cdh4	GACATCGGGGAGTTCATCAC	GGTCTGCCAGCTTTTTGAAG
(VE-)cdh5	ACACAAGATCCACACGCTGG	GAACATACACTCAGGAGCGTG
cdh13	CTCCGTATGTGTTCCCGTCT	GCAACATGATCTGGGAGTGA
pcdh1a	GGACCACTACGAAAGGACCA	TGATAAGGCACAAACGTGGA
pcdh1b	GCTCCAACCTCAATCACCAT	GGCTGTGGAGAAGGTCACTC
pcdh7a	TAGTGGGGTGGAGGACTCAG	CATTCGTGTGTGCATTTTCC
pcdh7b	TACAGCAGCCAAACCATCAA	CGGGCGTAGTCCTCTCATAG
pcdh10a	TCCAACGGGAGCATTTTATC	GCACTCTTCAGTGCAGTTGG
pcdh10b	GGGGGATCAGTCATTCTCAA	GGTATGTCCACTCGGAAGGA
pcdh18a	CCAGCTTCAGAGGGAACAAA	GGCACTCCTCCGTACACAAT
pcdh18b	TGCTTGGAGAAGGCTTCAGT	GCACTTCAGGAGTGGCTTTC
cdh4	GACATCGGGGAGTTCATCAC	GGTCTGCCAGCTTTTTGAAG
scla	CTGAAATCCGAGCAATTTCC	GTTTCCTTGGCAACACCATT
esr1	CAGGACCAGCCCGATTCC	TTAGGGTACATGGGTGAGAGTTTG
esr2a	CTCACAGCACGGACCCTAAAC	GGTTGTCCATCCTCCCGAAAC
esr2b	CGCTCGGCATGGACAAC	CCCATGCGGTGGAGAGTAAT

Transgenic Zebrafish Lines

Official Line Name	***Common Name***	***Reference***
Tg(hsp70l:vegfaa,myl7:EGFP)	*hs:vegfAa*	(Wiley et al., 2011)
Tg(-1.5hsp70l:Gal4)	*hs:Gal4*	(Scheer et al., 2002)
Tg(5xUAS-E1b:6xMYC-notch1a)	*UAS:NICD*	(Scheer and Campos-Ortega, 1999)
Tg(EPV.Tp1-Mmu.Hbb:EGFP)	*Notch:GFP*	(Parsons et al., 2009)
Tg(5xERE:GFP)	*ERE:GFP*	(Gorelick and Halpern, 2011)
Tg(runx1P1:eGFP)	*runx1:GFP*	(Lam et al., 2010)
Tg(-6.0itga2b (CD41):EGFP	*CD41:GFP*	(Ma et al., 2011)
Tg(kdrl:EGFP)	*flk:GFP*	(Beis et al., 2005)
Tg(cdh17:mCherry)	*cdh17:mCherry*	(Diep et al., 2011)
Tg(cmyb:EGFP)	*cmyb:GFP*	(North et al., 2007)
Tg(kdrl:DsRed2)	*flk:dsRed*	(Kikuchi et al., 2011)
mindbomb		(Itoh et al., 2003)
Tg(Tie2:EGFP)	*tie2:GFP*	(Motoike et al., 2000)
Tg(-6tal1:EGFP)	*scl:GFP*	(Zhang and Rodaway, 2007)
plcg1$^{y13/y13}$		(Covassin et al., 2009)
kdrl$^{y17/y17}$		(Covassin et al., 2006)
Tg(lmo2:dsRed)	*lmo2:dsRed*	(Zhu et al., 2005)
Tg(gata1a:GFP)	*gata1:gfp*	(Long et al., 1997)
Tg(hbbe1.1EGFP)	*globin:GFP*	*L.I. Zon Unpublished*
Tg(mpx:GFP)	*MPO:GFP*	(Mathias et al., 2006)
Tg(rag2:dsRed)	*rag2:dsRed*	(Ma et al., 2012)
Tg(spi1b:GAL4,UAS:EGFP)	*pu.1:GFP*	(Peri and Nüsslein-Volhard, 2008)
Tg(ptprc:DsRed)	*CD45:dsRed*	(Bertrand et al., 2008)
Tg(-10.0myf5:EGFP)	*myf5:GFP*	(Chen et al., 2007)

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